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(54) Title: MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

(57) Abstract: The invention provides modified virus Ankara (MVA), a replication-deficient strain of vaccinia virus, expressing human immunodeficiency virus (HIV) *env*, *gag*, and *pol* genes.

MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

Field of the Invention

The invention provides modified vaccinia Ankara (MVA), a replication-deficient strain of vaccinia virus, expressing human immunodeficiency virus (HIV) *env*, *gag*, and *pol* genes.

Background of the Invention

Cellular immunity plays an important role in the control of immunodeficiency virus infections (P.J. Goulder *et al.* 1999 *AIDS* **13**:S121). Recently, a DNA vaccine designed to enhance cellular immunity by cytokine augmentation successfully contained a highly virulent immunodeficiency virus challenge (D.H. Barouch *et al.* 2000 *Science* **290**:486). Another promising approach to raising cellular immunity is DNA priming followed by recombinant poxvirus boosters (H.L. Robinson *et al.* 2000 *AIDS Rev* **2**:105). This heterologous prime/boost regimen induces 10- to 100-fold higher frequencies of T cells than priming and boosting with DNA or recombinant poxvirus vaccines alone. Previously, investigators showed that boosting a DNA-primed response with a poxvirus was superior to boosting with DNA or protein for the control of a non-pathogenic immunodeficiency virus (H.L. Robinson *et al.* 1999 *Nat Med* **5**:526). There is a need for the control of a pathogenic immunodeficiency virus.

Summary of the Invention

Here we report that DNA priming followed by a recombinant modified vaccinia Ankara (rMVA) booster has controlled a highly pathogenic immunodeficiency virus challenge in a rhesus macaque model. Both the DNA and rMVA components of the vaccine expressed multiple immunodeficiency virus proteins. Two DNA inoculations at 0 and 8 weeks and a single rMVA booster at 24 weeks effectively controlled an intrarectal challenge administered seven months after the booster. These findings are envisioned as indicating that a relatively simple multiprotein DNA/MVA vaccine can help to control the acquired immune deficiency syndrome (AIDS) epidemic. We also report that inoculations of rMVA induce good immune responses even without DNA priming.

Brief Description of the Drawings

Figure I. Phylogenetic relationships of HIV-1 and HIV-2 based on identity of *pol* gene sequences. SIV_{cpz} and SIV_{smm} are subhuman primate lentiviruses recovered from a chimpanzee and sooty mangabey monkey, respectively.

Figure II. Phylogenetic relationships of HIV-1 groups M, N and O with four different SIV_{cpz} isolates based on full-length *pol* gene sequences. The bar indicates a genetic distance of 0.1 (10% nucleotide divergence) and the *asterisk* positions group N HIV-1 isolates based on *env* sequences.

Figure III. Tropic and biologic properties of HIV-1 isolates.

Figure IV. HIV-encoded proteins. The location of the HIV genes, the sizes of primary translation products (in some cases polyproteins), and the processed mature viral proteins are indicated.

Figure V. Schematic representation of a mature HIV-1 virion.

Figure VI. Linear representation of the HIV-1 Env glycoprotein. The *arrow* indicates the site of gp160 cleavage to gp120 and gp41. In gp120, *cross-hatched* areas represent variable domains (V_1 to V_5) and *open boxes* depict conserved sequences (C_1 to C_5). In the gp41 ectodomain, several domains are indicated: the N-terminal fusion peptide, and the two ectodomain helices (N- and C-helix). The membrane-spanning domain is represented by a *black box*. In the gp41 cytoplasmic domain, the Tyr-X-X-Leu (YXXL) endocytosis motif (SEQ ID NO: 9) and two predicted helical domains (helix-1 and –2) are shown. Amino acid numbers are indicated.

Figure 1. Temporal frequencies of Gag-specific T cells. (**A**) Gag-specific CD8 T cell responses raised by DNA priming and rMVA booster immunizations. The schematic presents mean Gag-CM9-tetramer data generated in the high-dose i.d. DNA-immunized animals. (**B**) Gag–specific IFN-γ ELISPOTs in *A*01* (open bars) and non-*A*01* (filled bars) macaques at various times before challenge and at two weeks after challenge. Three pools of 10 to 13 Gag peptides (22-mers overlapping by 12) were used for the analyses. The numbers above data bars represent the arithmetic mean ± SD for the ELISPOTs within each group. The numbers at the top of the graphs designate individual animals. *, data not available; #, <20 ELISPOTs per $1x10^6$ peripheral blood mononuclear cells (PBMC). Temporal data for Gag-CM9-Mamu-A*01 tetramer-specific T cells can be found in Figure 6.

Figure 2. Temporal viral loads, CD4 counts, and survival after challenge of vaccinated and control animals. (**A**) Geometric mean viral loads and (**B**) geometric mean CD4 counts. (**C**) Survival curve for vaccinated and control animals. The dotted line represents all 24 vaccinated animals. (**D**) Viral loads and (**E**) CD4 counts for individual animals in the vaccine and control groups. The key to animal numbers is presented in (**E**). Assays for the first 12 weeks after challenge had a detection level of 1000 copies of RNA per milliliter of plasma. Animals with loads below 1000 were scored with a load of 500. For weeks 16 and 20, the detection level was 300 copies of RNA per milliliter. Animals with levels of virus below 300 were scored at 300.

Figure 3. Postchallenge T cell responses in vaccine and control groups. (**A**) Temporal tetramer$^+$ cells (dashed line) and viral loads (solid line). (**B**) Intracellular cytokine assays for IFN-γ production in response to stimulation with the Gag-CM9 peptide at two weeks after challenge. This *ex vivo* assay allows evaluation of the functional status of the peak postchallenge tetramer$^+$ cells displayed in Figure 1A. (**C**) Proliferation assay at 12 weeks after challenge. Gag-Pol-Env (open bars) and Gag-Pol (hatched bars) produced by transient transfections were used for stimulation. Supernatants from mock-transfected cultures served as control antigen. Stimulation indices are the growth of cultures in the presence of viral antigens divided by the growth of cultures in the presence of mock antigen.

Figure 4. Lymph node histomorphology at 12 weeks after challenge. (**A**) Typical lymph node from a vaccinated macaque showing evidence of follicular hyperplasia characterized by the presence of numerous secondary follicles with expanded germinal centers and discrete dark and light zones. (**B**) Typical lymph node from an infected control animal showing follicular depletion and paracortical lymphocellular atrophy. (**C**) A representative lymph node from an age-matched, uninfected macaque displaying nonreactive germinal centers. (**D**) The percentage of the total lymph node area occupied by germinal centers was measured to give a non-specific indicator of follicular hyperplasia. Data for uninfected controls are for four age-matched rhesus macaques.

Figure 5. Temporal antibody responses. Micrograms of total Gag (**A**) or Env (**B**) antibody were determined with ELISAs. The titers of neutralizing antibody for SHIV-89.6 (**C**) and SHIV-89.6P (**D**) were determined with MT-2 cell killing and neutral red staining (D.C. Montefiori *et al.* 1988 *J Clin Microbiol* **26**:231). Titers are the reciprocal of the

serum dilution giving 50% neutralization of the indicated viruses grown in human PBMC. Symbols for animals are the same as in Figure 2.

Figure 6. Gag-CM9-Mamu-A*01 tetramer-specific T cells in *Mamu-A*01* vaccinated and control macaques at various times before challenge and at two weeks after challenge. The number at the upper right corner of each plot represents the frequency of tetramer-specific CD8 T cells as a % of total CD8 T cells. The numbers above each column of FACS data designate individual animals.

Figure A. Map and sequence of plasmid transfer vector pLW-48.

Figure B. Sequences of plasmid transfer vector pLW-48, Psy II promoter (which controls ADA envelope expression), ADA envelope truncated, PmH5 promoter (which controls HXB2 gag pol expression), and HXB2 *gag pol* (with safety mutations, Δ integrase).

Figure C. Plasmid transfer vector pLW-48 and making MVA recombinant virus MVA/HIV 48.

Figure D. A clade B gag pol.

Figure E. Sequence of new Psyn II promoter.

Detailed Description of the Preferred Embodiment

Recombinant MVA Virus

Vaccinia virus, a member of the genus Orthopoxvirus in the family of Poxviridae, was used as live vaccine to immunize against the human smallpox disease. Successful worldwide vaccination with vaccinia virus culminated in the eradication of variola virus, the causative agent of the smallpox (The global eradication of smallpox. Final report of the global commission for the certification of smallpox eradication. History of Public Health, No. 4, Geneva: World Health Organization, 1980). Since that WHO declaration, vaccination has been universally discontinued except for people at high risk of poxvirus infections (e.g. laboratory workers).

More recently, vaccinia viruses have also been used to engineer viral vectors for recombinant gene expression and for the potential use as recombinant live vaccines (Mackett, M. *et al.* 1982 *PNAS USA* 79:7415-7419; Smith, G.L. *et al.* 1984 Biotech Genet Engin Rev 2:383-407). This entails DNA sequences (genes) which code for foreign antigens being introduced, with the aid of DNA recombination techniques, into the genome of the vaccinia viruses. If the gene is integrated at a site in the viral DNA which is non-

essential for the life cycle of the virus, it is possible for the newly produced recombinant vaccinia virus to be infectious, that is to say able to infect foreign cells and thus to express the integrated DNA sequence (EP Patent Applications No. 83,286 and No. 110,385). The recombinant vaccinia viruses prepared in this way can be used, on the one hand, as live vaccines for the prophylaxis of infectious diseases, on the other hand, for the preparation of heterologous proteins in eukaryotic cells.

For vector applications health risks would be lessened by the use of a highly attenuated vaccinia virus strain. Several such strains of vaccinia virus were especially developed to avoid undesired side effects of smallpox vaccination. Thus, the modified vaccinia Ankara (MVA) has been generated by long-term serial passages of the Ankara strain of vaccinia virus (CVA) on chicken embryo fibroblasts (for review see Mayr, A. *et al.* 1975 *Infection* 3:6-14; Swiss Patent No. 568,392). The MVA virus is publicly available from American Type Culture Collection as ATCC No. VR-1508. MVA is distinguished by its great attenuation, that is to say by diminished virulence and ability to replicate in primate cells while maintaining good immunogenicity. The MVA virus has been analyzed to determine alterations in the genome relative to the parental CVA strain. Six major deletions of genomic DNA (deletion I, II, III, IV, V, and VI) totaling 31,000 base pairs have been identified (Meyer, H. *et al.* 1991 *J Gen Virol* **72**:1031-1038). The resulting MVA virus became severely host cell restricted to avian cells.

Furthermore, MVA is characterized by its extreme attenuation. When tested in a variety of animal models, MVA was proven to be avirulent even in immunosuppressed animals. More importantly, the excellent properties of the MVA strain have been demonstrated in extensive clinical trials (Mayr A. *et al.* 1978 Zentralbl Bakteriol [B] **167**:375-390; Stickl *et al.* 1974 Dtsch Med Wschr **99**:2386-2392). During these studies in over 120,000 humans, including high-risk patients, no side effects were associated with the use of MVA vaccine.

MVA replication in human cells was found to be blocked late in infection preventing the assembly to mature infectious virions. Nevertheless, MVA was able to express viral and recombinant genes at high levels even in non-permissive cells and was proposed to serve as an efficient and exceptionally safe gene expression vector (Sutter, G. and Moss, B. 1992 *PNAS USA* **89**:10847-10851). Additionally, novel vaccinia vector vaccines were established on the basis of MVA having foreign DNA sequences inserted at

the site of deletion III within the MVA genome (Sutter, G. *et al.* 1994 Vaccine **12**:1032-1040).

The recombinant MVA vaccinia viruses can be prepared as set out hereinafter. A DNA-construct which contains a DNA-sequence which codes for a foreign polypeptide flanked by MVA DNA sequences adjacent to a naturally occurring deletion, e.g. deletion III, or other non-essential sites, within the MVA genome, is introduced into cells infected with MVA, to allow homologous recombination. Once the DNA-construct has been introduced into the eukaryotic cell and the foreign DNA has recombined with the viral DNA, it is possible to isolate the desired recombinant vaccinia virus in a manner known per se, preferably with the aid of a marker. The DNA-construct to be inserted can be linear or circular. A plasmid or polymerase chain reaction product is preferred. The DNA-construct contains sequences flanking the left and the right side of a naturally occurring deletion, e.g. deletion III, within the MVA genome. The foreign DNA sequence is inserted between the sequences flanking the naturally occurring deletion. For the expression of a DNA sequence or gene, it is necessary for regulatory sequences, which are required for the transcription of the gene, to be present on the DNA. Such regulatory sequences (called promoters) are known to those skilled in the art, and include for example those of the vaccinia 11 kDa gene as are described in EP-A-198,328, and those of the 7.5 kDa gene (EP-A-110,385). The DNA-construct can be introduced into the MVA infected cells by transfection, for example by means of calcium phosphate precipitation (Graham *et al.* 1973 *Virol* **52**:456-467; Wigler *et al.* 1979 *Cell* **16**:777-785), by means of electroporation (Neumann *et al.* 1982 *EMBO J* **1**:841-845), by microinjection (Graessmann *et al.* 1983 *Meth Enzymol* **101**:482-492), by means of liposomes (Straubinger *et al.* 1983 *Meth Enzymol* **101**:512-527), by means of spheroplasts (Schaffner 1980 *PNAS USA* **77**:2163-2167) or by other methods known to those skilled in the art.

HIVs and Their Replication

The etiological agent of acquired immune deficiency syndrome (AIDS) is recognized to be a retrovirus exhibiting characteristics typical of the lentivirus genus, referred to as human immunodeficiency virus (HIV). The phylogenetic relationships of the human lentiviruses are shown in Figure I. HIV-2 is more closely related to SIV_{smm}, a virus isolated from sooty mangabey monkeys in the wild, than to HIV-l. It is currently believed

that HIV-2 represents a zoonotic transmission of SIV_{smm} to man. A series of lentiviral isolates from captive chimpanzees, designated SIV_{cpz}, are close genetic relatives of HIV-l.

The earliest phylogenetic analyses of HIV-1 isolates focused on samples from Europe/North America and Africa; discrete clusters of viruses were identified from these two areas of the world. Distinct genetic subtypes or clades of HIV-l were subsequently defined and classified into three groups: M (major); O (outlier); and N (non-M or O) (Fig. II). The M group of HIV-l, which includes over 95% of the global virus isolates, consists of at least eight discrete clades (A, B, C, D, F, G, H, and J), based on the sequence of complete viral genomes. Members of HIV-l group O have been recovered from individuals living in Cameroon, Gabon, and Equatorial Guinea; their genomes share less than 50% identity in nucleotide sequence with group M viruses. The more recently discovered group N HIV-I strains have been identified in infected Cameroonians, fail to react serologically in standard whole-virus enzyme-linked immunosorbent assay (ELISA), yet are readily detectable by conventional Western blot analysis.

Most current knowledge about HIV-l genetic variation comes from studies of group M viruses of diverse geographic origin. Data collected during the past decade indicate that the HIV-1 population present within an infected individual can vary from 6% to 10% in nucleotide sequence. HIV-l isolates within a clade may exhibit nucleotide distances of 15% in *gag* and up to 30% in gp120 coding sequences. Interclade genetic variation may range between 30% and 40% depending on the gene analyzed.

All of the HIV-1 group M subtypes can be found in Africa. Clade A viruses are genetically the most divergent and were the most common HIV-1 subtype in Africa early in the epidemic. With the rapid spread of HIV-l to southern Africa during the mid to late 1990s, clade C viruses have become the dominant subtype and now account for 48% of HIV-l infections worldwide. Clade B viruses, the most intensively studied HIV-1 subtype, remain the most prevalent isolates in Europe and North America.

High rates of genetic recombination are a hallmark of retroviruses. It was initially believed that simultaneous infections by genetically diverse virus strains were not likely to be established in individuals at risk for HIV-1. By 1995, however, it became apparent that a significant fraction of the HIV-1 group M global diversity included interclade viral recombinants. It is now appreciated that HIV-1 recombinants will be found in geographic areas such as Africa, South America, and Southeast Asia, where multiple HIV-1 subtypes

coexist and may account for more than 10% of circulating HIV-1 strains. Molecularly, the genomes of these recombinant viruses resemble patchwork mosaics, with juxtaposed diverse HIV-1 subtype segments, reflecting the multiple crossover events contributing to their generation. Most HIV-1 recombinants have arisen in Africa and a majority contain segments originally derived from clade A viruses. In Thailand, for example, the composition of the predominant circulating strain consists of a clade A *gag* plus *pol* gene segment and a clade E *env* gene. Because the clade E *env* gene in Thai HIV-1 strains is closely related to the clade E *env* present in virus isolates from the Central African Republic, it is believed that the original recombination event occurred in Africa, with the subsequent introduction of a descendent virus into Thailand. Interestingly, no full-length HIV-1 subtype E isolate (i.e., with subtype E *gag*, *pol*, and *env* genes) has been reported to date.

The discovery that α and β chemokine receptors function as coreceptors for virus fusion and entry into susceptible $CD4^+$ cells has led to a revised classification scheme for HIV-1 (Fig. III). Isolates can now be grouped on the basis of chemokine receptor utilization in fusion assays in which HIV-1 gp120 and $CD4^+$ coreceptor proteins are expressed in separate cells. As indicated in Figure III, HIV-1 isolates using the CXCR4 receptor (now designated X4 viruses) are usually T cell line (TCL)-tropic syncytium inducing (SI) strains, whereas those exclusively utilizing the CCR5 receptor (R5 viruses) are predominantly macrophage (M)-tropic and non-syncytium inducing (NSI). The dual-tropic R5/X4 strains, which may comprise the majority of patient isolates and exhibit a continuum of tropic phenotypes, are frequently SI.

As is the case for all replication-competent retroviruses, the three primary HIV-1 translation products, all encoding structural proteins, are initially synthesized as polyprotein precursors, which are subsequently processed by viral or cellular proteases into mature particle-associated proteins (Fig. IV). The 55-kd Gag precursor $Pr55^{Gag}$ is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. Autocatalysis of the 160-kd Gag-Pol polyprotein, $Pr160^{Gag\text{-}Pol}$, gives rise to the protease (PR), the heterodimeric reverse transcriptase (RT), and the integrase (IN) proteins, whereas proteolytic digestion by a cellular enzyme(s) converts the glycosylated 160-kd Env precursor gp160 to the gp120 surface (SU) and gp41 transmembrane (TM) cleavage products. The remaining six HIV-1-

encoded proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef) are the primary translation products of spliced mRNAs.

Gag

The Gag proteins of HIV, like those of other retroviruses, are necessary and sufficient for the formation of noninfectious, virus-like particles. Retroviral Gag proteins are generally synthesized as polyprotein precursors; the HIV-l Gag precursor has been named, based on its apparent molecular mass, $Pr55^{Gag}$. As noted previously, the mRNA for $Pr55^{Gag}$ is the unspliced 9.2-kb transcript (Fig. IV) that requires Rev for its expression in the cytoplasm. When the *pol* ORF is present, the viral protease (PR) cleaves $Pr55^{Gag}$ during or shortly after budding from the cell to generate the mature Gag proteins p17 (MA), p24 (CA), p7 (NC), and p6 (see Fig. IV). In the virion, MA is localized immediately inside the lipid bilayer of the viral envelope, CA forms the outer portion of the cone-shaped core structure in the center of the particle, and NC is present in the core in a ribonucleoprotein complex with the viral RNA genome (Fig. V).

The HIV $Pr55^{Gag}$ precursor oligomerizes following its translation and is targeted to the plasma membrane, where particles of sufficient size and density to be visible by EM are assembled. Formation of virus-like particles by $Pr55^{Gag}$ is a self-assembly process, with critical Gag-Gag interactions taking place between multiple domains along the Gag precursor. The assembly of virus-like particles does not require the participation of genomic RNA (although the presence of nucleic acid appears to be essential), *pol*-encoded enzymes, or Env glycoproteins, but the production of infectious virions requires the encapsidation of the viral RNA genome and the incorporation of the Env glycoproteins and the Gag-Pol polyprotein precursor $Pr160^{Gag\text{-}Pol}$.

Pol

Downstream of *gag* lies the most highly conserved region of the HIV genome, the *pol* gene, which encodes three enzymes: PR, RT, and IN (see Fig. IV). RT and IN are required, respectively, for reverse transcription of the viral RNA genome to a double-stranded DNA copy, and for the integration of the viral DNA into the host cell chromosome. PR plays a critical role late in the life cycle by mediating the production of mature, infectious virions. The *pol* gene products are derived by enzymatic cleavage of a 160-kd Gag-Pol fusion protein, referred to as $Pr160^{Gag\text{-}Pol}$. This fusion protein is produced by ribosomal frameshifting during translation of $Pr55^{Gag}$ (see Fig. IV). The frame-shifting

mechanism for Gag-Pol expression, also utilized by many other retroviruses, ensures that the *pol*-derived proteins are expressed at a low level, approximately 5% to 10% that of Gag. Like $Pr55^{Gag}$, the N-terminus of $Pr160^{Gag\text{-}Pol}$ is myristylated and targeted to the plasma membrane.

Protease

Early pulse-chase studies performed with avian retroviruses clearly indicated that retroviral Gag proteins are initially synthesized as polyprotein precursors that are cleaved to generate smaller products. Subsequent studies demonstrated that the processing function is provided by a viral rather than a cellular enzyme, and that proteolytic digestion of the Gag and Gag-Pol precursors is essential for virus infectivity. Sequence analysis of retroviral PRs indicated that they are related to cellular "aspartic" proteases such as pepsin and renin. Like these cellular enzymes, retroviral PRs use two apposed Asp residues at the active site to coordinate a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. Unlike the cellular aspartic proteases, which function as pseudodimers (using two folds within the same molecule to generate the active site), retroviral PRs function as true dimers. X-ray crystallographic data from HIV-1 PR indicate that the two monomers are held together in part by a four-stranded antiparallel β-sheet derived from both N- and C-terminal ends of each monomer. The substrate-binding site is located within a cleft formed between the two monomers. Like their cellular homologs, the HIV PR dimer contains flexible "flaps" that overhang the binding site and may stabilize the substrate within the cleft; the active-site Asp residues lie in the center of the dimer. Interestingly, although some limited amino acid homology is observed surrounding active-site residues, the primary sequences of retroviral PRs are highly divergent, yet their structures are remarkably similar.

Reverse Transcriptase

By definition, retroviruses possess the ability to convert their single-stranded RNA genomes into double-stranded DNA during the early stages of the infection process. The enzyme that catalyzes this reaction is RT, in conjunction with its associated RNaseH activity. Retroviral RTs have three enzymatic activities: (a) RNA-directed DNA polymerization (for minus-strand DNA synthesis), (b) RNaseH activity (for the degradation of the tRNA primer and genomic RNA present in DNA-RNA hybrid intermediates), and (c) DNA-directed DNA polymerization (for second- or plus-strand DNA synthesis).

The mature HIV-1 RT holoenzyme is a heterodimer of 66 and 51 kd subunits. The 51-kd subunit (p51) is derived from the 66-kd (p66) subunit by proteolytic removal of the C-terminal 15-kd RNaseH domain of p66 by PR (see Fig. IV). The crystal structure of HIV-1 RT reveals a highly asymmetric folding in which the orientations of the p66 and p51 subunits differ substantially. The p66 subunit can be visualized as a right hand, with the polymerase active site within the palm, and a deep template-binding cleft formed by the palm, fingers, and thumb subdomains. The polymerase domain is linked to RNaseH by the connection subdomain. The active site, located in the palm, contains three critical Asp residues (110, 185, and 186) in close proximity, and two coordinated Mg^{2+} ions. Mutation of these Asp residues abolishes RT polymerizing activity. The orientation of the three active-site Asp residues is similar to that observed in other DNA polymerases (e.g., the Klenow fragment of *E. coli* DNA polI). The p51 subunit appears to be rigid and does not form a polymerizing cleft; Asp 110, 185, and 186 of this subunit are buried within the molecule. Approximately 18 base pairs of the primer-template duplex lie in the nucleic acid binding cleft, stretching from the polymerase active site to the RNaseH domain.

In the RT-primer-template-dNTP structure, the presence of a dideoxynucleotide at the 3' end of the primer allows visualization of the catalytic complex trapped just prior to attack on the incoming dNTP. Comparison with previously obtained structures suggests a model whereby the fingers close in to trap the template and dNTP prior to nucleophilic attack of the 3'-OH of the primer on the incoming dNTP. After the addition of the incoming dNTP to the growing chain, it has been proposed that the fingers adopt a more open configuration, thereby releasing the pyrophosphate and enabling RT to bind the next dNTP. The structure of the HIV-1 RNaseH has also been determined by x-ray crystallography; this domain displays a global folding similar to that of *E. coli* RNaseH.

Integrase

A distinguishing feature of retrovirus replication is the insertion of a DNA copy of the viral genome into the host cell chromosome following reverse transcription. The integrated viral DNA (the provirus) serves as the template for the synthesis of viral RNAs and is maintained as part of the host cell genome for the lifetime of the infected cell. Retroviral mutants deficient in the ability to integrate generally fail to establish a productive infection.

The integration of viral DNA is catalyzed by integrase, a 32-kd protein generated by PR-mediated cleavage of the C-terminal portion of the HIV-1 Gag-Pol polyprotein (see Fig. IV).

Retroviral IN proteins are composed of three structurally and functionally distinct domains: an N-terminal, zinc-finger-containing domain, a core domain, and a relatively nonconserved C-terminal domain. Because of its low solubility, it has not yet been possible to crystallize the entire 288-amino-acid HIV-1 IN protein. However, the structure of all three domains has been solved independently by x-ray crystallography or NMR methods. The crystal structure of the core domain of the avian sarcoma virus IN has also been determined. The N-terminal domain (residues 1 to 55), whose structure was solved by NMR spectroscopy, is composed of four helices with a zinc coordinated by amino acids His-12, His-16, Cys-40, and Cys-43. The structure of the N-terminal domain is reminiscent of helical DNA binding proteins that contain a so-called helix-turn-helix motif; however, in the HIV-1 structure this motif contributes to dimer formation. Initially, poor solubility hampered efforts to solve the structure of the core domain. However, attempts at crystallography were successful when it was observed that a Phe-to-Lys change at IN residue 185 greatly increased solubility without disrupting *in vitro* catalytic activity. Each monomer of the HIV-1 IN core domain (IN residues 50 to 212) is composed of a five-stranded β-sheet flanked by helices; this structure bears striking resemblance to other polynucleotidyl transferases including RNaseH and the bacteriophage MuA transposase. Three highly conserved residues are found in analogous positions in other polynucleotidyl transferases; in HIV-1 IN these are Asp-64, Asp-116 and Glu-152, the so-called D,D-35-E motif. Mutations at these positions block HIV IN function both *in vivo* and *in vitro*. The close proximity of these three amino acids in the crystal structure of both avian sarcoma virus and HIV-1 core domains supports the hypothesis that these residues play a central role in catalysis of the polynucleotidyl transfer reaction that is at the heart of the integration process. The C-terminal domain, whose structure has been solved by NMR methods, adopts a five-stranded β-barrel folding topology reminiscent of a Src homology 3 (SH3) domain. Recently, the x-ray structures of SIV and Rous sarcoma virus IN protein fragments encompassing both the core and C-terminal domains have been solved.

Env

The HIV Env glycoproteins play a major role in the virus life cycle. They contain the determinants that interact with the CD4 receptor and coreceptor, and they catalyze the fusion reaction between the lipid bilayer of the viral envelope and the host cell plasma membrane. In addition, the HIV Env glycoproteins contain epitopes that elicit immune responses that are important from both diagnostic and vaccine development perspectives.

The HIV Env glycoprotein is synthesized from the singly spliced 4.3-kb Vpu/Env bicistronic mRNA (see Fig. IV); translation occurs on ribosomes associated with the rough endoplasmic reticulum (ER). The 160-kd polyprotein precursor (gp160) is an integral membrane protein that is anchored to cell membranes by a hydrophobic stop-transfer signal in the domain destined to be the mature TM Env glycoprotein, gp41 (Fig. VI). The gp160 is cotranslationally glycosylated, forms disulfide bonds, and undergoes oligomerization in the ER. The predominant oligomeric form appears to be a trimer, although dimers and tetramers are also observed. The gp160 is transported to the Golgi, where, like other retroviral envelope precursor proteins, it is proteolytically cleaved by cellular enzymes to the mature SU glycoprotein gp120 and TM glycoprotein gp41 (see Fig. VI). The cellular enzyme responsible for cleavage of retroviral Env precursors following a highly conserved Lys/Arg-X-Lys/Arg-Arg motif is furin or a furin-like protease, although other enzymes may also catalyze gp160 processing. Cleavage of gp160 is required for Env-induced fusion activity and virus infectivity. Subsequent to gp160 cleavage, gp120 and gp41 form a noncovalent association that is critical for transport of the Env complex from the Golgi to the cell surface. The gp120-gp41 interaction is fairly weak, and a substantial amount of gp120 is shed from the surface of Env-expressing cells.

The HIV Env glycoprotein complex, in particular the SU (gp120) domain, is very heavily glycosylated; approximately half the molecular mass of gp160 is composed of oligosaccharide side chains. During transport of Env from its site of synthesis in the ER to the plasma membrane, many of the side chains are modified by the addition of complex sugars. The numerous oligosaccharide side chains form what could be imagined as a sugar cloud obscuring much of gp120 from host immune recognition. As shown in Figure VI, gp120 contains interspersed conserved (C_1 to C_5) and variable (V_1 to V_5) domains. The Cys residues present in the gp120s of different isolates are highly conserved and form disulfide bonds that link the first four variable regions in large loops.

A primary function of viral Env glycoproteins is to promote a membrane fusion reaction between the lipid bilayers of the viral envelope and host cell membranes. This membrane fusion event enables the viral core to gain entry into the host cell cytoplasm. A number of regions in both gp120 and gp41 have been implicated, directly or indirectly, in Env-mediated membrane fusion. Studies of the HA_2 hemagglutinin protein of the orthomyxoviruses and the F protein of the paramyxoviruses indicated that a highly hydrophobic domain at the N-terminus of these proteins, referred to as the fusion peptide, plays a critical role in membrane fusion. Mutational analyses demonstrated that an analogous domain was located at the N-terminus of the HIV-l, HIV-2, and SIV TM glycoproteins (see Fig. VI). Nonhydrophobic substitutions within this region of gp41 greatly reduced or blocked syncytium formation and resulted in the production of noninfectious progeny virions.

C-terminal to the gp41 fusion peptide are two amphipathic helical domains (see Fig. VI) which play a central role in membrane fusion. Mutations in the N-terminal helix (referred to as the N-helix), which contains a Leu zipper-like heptad repeat motif, impair infectivity and membrane fusion activity, and peptides derived from these sequences exhibit potent antiviral activity in culture. The structure of the ectodomain of HIV-l and SIV gp41, the two helical motifs in particular, has been the focus of structural analyses in recent years. Structures were determined by x-ray crystallography or NMR spectroscopy either for fusion proteins containing the helical domains, a mixture of peptides derived from the N- and C-helices, or in the case of the SIV structure, the intact gp41 ectodomain sequence from residue 27 to 149. These studies obtained fundamentally similar trimeric structures, in which the two helical domains pack in an antiparallel fashion to generate a six-helix bundle. The N-helices form a coiled-coil in the center of the bundle, with the C-helices packing into hydrophobic grooves on the outside.

In the steps leading to membrane fusion CD4 binding induces conformation changes in Env that facilitate coreceptor binding. Following the formation of a ternary gp120/CD4/coreceptor complex, gp41 adopts a hypothetical conformation that allows the fusion peptide to insert into the target lipid bilayer. The formation of the gp41 six-helix bundle (which involves antiparallel interactions between the gp41 N- and C-helices) brings the viral and cellular membranes together and membrane fusion takes place.

Use of Recombinant MVA Virus To Boost CD+8 Cell Immune Response

The present invention relates to generation of a $CD8^+$ T cell immune response against an antigen and also eliciting an antibody response. More particularly, the present invention relates to "prime and boost" immunization regimes in which the immune response induced by administration of a priming composition is boosted by administration of a boosting composition. The present invention is based on inventors' experimental demonstration that effective boosting can be achieved using modified vaccinia Ankara (MVA) vectors, following priming with any of a variety of different types of priming compositions including recombinant MVA itself.

A major protective component of the immune response against a number of pathogens is mediated by T lymphocytes of the $CD8^+$ type, also known as cytotoxic T lymphocytes (CTL). An important function of $CD8^+$ cells is secretion of gamma interferon (IFNγ), and this provides a measure of $CD8^+$ T cell immune response. A second component of the immune response is antibody directed to the proteins of the pathogen.

The present invention employs MVA which, as the experiments described below show, has been found to be an effective means for providing a boost to a $CD8^+$ T cell immune response primed to antigen using any of a variety of different priming compositions and also eliciting an antibody response.

Remarkably, the experimental work described below demonstrates that use of embodiments of the present invention allows for recombinant MVA virus expressing an HIV antigen to boost a $CD8^+$ T cell immune response primed by a DNA vaccine and also eliciting an antibody response. The MVA was found to induce a $CD8^+$ T cell response after intradermal, intramuscular or mucosal immunization. Recombinant MVA has also been shown to prime an immune response that is boosted by one or more inoculations of recombinant MVA.

Non-human primates immunized with plasmid DNA and boosted with the MVA were effectively protected against intramucosal challenge with live virus. Advantageously, the inventors found that a vaccination regime used intradermal, intramuscular or mucosal immunization for both prime and boost can be employed, constituting a general immunization regime suitable for inducing $CD8^+$ T cells and also eliciting an antibody response, e.g. in humans.

The present invention in various aspects and embodiments employs an MVA vector encoding an HIV antigen for boosting a $CD8^+$ T cell immune response to the antigen primed by previous administration of nucleic acid encoding the antigen and also eliciting an antibody response.

A general aspect of the present invention provides for the use of an MVA vector for boosting a $CD8^+$ T cell immune response to an HIV antigen and also eliciting an antibody response.

One aspect of the present invention provides a method of boosting a $CD8^+$ T cell immune response to an HIV antigen in an individual, and also eliciting an antibody response, the method including provision in the individual of an MVA vector including nucleic acid encoding the antigen operably linked to regulatory sequences for production of antigen in the individual by expression from the nucleic acid, whereby a $CD8^+$ T cell immune response to the antigen previously primed in the individual is boosted.

An immune response to an HIV antigen may be primed by immunization with plasmid DNA or by infection with an infectious agent.

A further aspect of the invention provides a method of inducing a $CD8^+$ T cell immune response to an HIV antigen in an individual, and also eliciting an antibody response, the method comprising administering to the individual a priming composition comprising nucleic acid encoding the antigen and then administering a boosting composition which comprises an MVA vector including nucleic acid encoding the antigen operably linked to regulatory sequences for production of antigen in the individual by expression from the nucleic acid.

A further aspect provides for use of an MVA vector, as disclosed, in the manufacture of a medicament for administration to a mammal to boost a $CD8^+$ T cell immune response to an HIV antigen, and also eliciting an antibody response. Such a medicament is generally for administration following prior administration of a priming composition comprising nucleic acid encoding the antigen.

The priming composition may comprise any viral vector, such as a vaccinia virus vector such as a replication-deficient strain such as modified vaccinia Ankara (MVA) or NYVAC (Tartaglia *et al.* 1992 *Virology* **118**:217-232), an avipox vector such as fowlpox or canarypox, e.g. the strain known as ALVAC (Paoletti *et al.* 1994 *Dev Biol Stand* **82**:65-69), or an adenovirus vector or a vesicular stomatitis virus vector or an alphavirus vector.

The priming composition may comprise DNA encoding the antigen, such DNA preferably being in the form of a circular plasmid that is not capable of replicating in mammalian cells. Any selectable marker should not be resistance to an antibiotic used clinically, so for example Kanamycin resistance is preferred to Ampicillin resistance. Antigen expression should be driven by a promoter which is active in mammalian cells, for instance the cytomegalovirus immediate early (CMV IE) promoter.

In particular embodiments of the various aspects of the present invention, administration of a priming composition is followed by boosting with a boosting composition, or first and second boosting compositions, the first and second boosting compositions being the same or different from one another. Still further boosting compositions may be employed without departing from the present invention. In one embodiment, a triple immunization regime employs DNA, then adenovirus as a first boosting composition, then MVA as a second boosting composition, optionally followed by a further (third) boosting composition or subsequent boosting administration of one or other or both of the same or different vectors. Another option is DNA then MVA then adenovirus, optionally followed by subsequent boosting administration of one or other or both of the same or different vectors.

The antigen to be encoded in respective priming and boosting compositions (however many boosting compositions are employed) need not be identical, but should share at least one $CD8^+$ T cell epitope. The antigen may correspond to a complete antigen, or a fragment thereof. Peptide epitopes or artificial strings of epitopes may be employed, more efficiently cutting out unnecessary protein sequence in the antigen and encoding sequence in the vector or vectors. One or more additional epitopes may be included, for instance epitopes which are recognized by T helper cells, especially epitopes recognized in individuals of different HLA types.

An HIV antigen of the invention to be encoded by a recombinant MVA virus includes polypeptides having immunogenic activity elicited by an amino acid sequence of an HIV Env, Gag, Pol, Vif, Vpr, Tat, Rev, Vpu, or Nef amino acid sequence as at least one $CD8^+$ T cell epitope. This amino acid sequence substantially corresponds to at least one 10-900 amino acid fragment and/or consensus sequence of a known HIV Env or Pol; or at least one 10-450 amino acid fragment and/or consensus sequence of a known HIV Gag; or at

least one 10-100 amino acid fragment and/or consensus sequence of a known HIV Vif, Vpr, Tat, Rev, Vpu, or Nef.

Although a full length Env precursor sequence is presented for use in the present invention, Env is optionally deleted of subsequences. For example, regions of the gp120 surface and gp41 transmembrane cleavage products can be deleted.

Although a full length Gag precursor sequence is presented for use in the present invention, Gag is optionally deleted of subsequences. For example, regions of the matrix protein (p17), regions of the capsid protein (p24), regions of the nucleocapsid protein (p7), and regions of p6 (the C-terminal peptide of the Gag polyprotein) can be deleted.

Although a full length Pol precursor sequence is presented for use in the present invention, Pol is optionally deleted of subsequences. For example, regions of the protease protein (p10), regions of the reverse transcriptase protein (p66/p51), and regions of the integrase protein (p32) can be deleted.

Such an HIV Env, Gag, or Pol can have overall identity of at least 50% to a known Env, Gag, or Pol protein amino acid sequence, such as 50-99% identity, or any range or value therein, while eliciting an immunogenic response against at least one strain of an HIV.

Percent identify can be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J Mol Biol* 1970 **48**:443), as revised by Smith and Waterman (*Adv Appl Math* 1981 **2**:482). Briefly, the GAP program defines identity as the number of aligned symbols (i.e., nucleotides or amino acids) which are identical, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unitary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess (*Nucl Acids Res* 1986 **14**:6745), as described by Schwartz and Dayhoff (eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, D.C. 1979, pp. 353-358); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

In a preferred embodiment, an Env of the present invention is a variant form of at least one HIV envelope protein. Preferably, the Env is composed of gp120 and the

membrane-spanning and ectodomain of gp41 but lacks part or all of the cytoplasmic domain of gp41.

Known HIV sequences are readily available from commercial and institutional HIV sequence databases, such as GENBANK, or as published compilations, such as Myers *et al.* eds., *Human* Retroviruses *and AIDS, A Compilation and Analysis of Nuc'eic Acid and Amino Acid Sequences*, Vol. I and II, Theoretical Biology and Biophysics, Los Alamos, N. Mex. (1993), or http://hiv-web.lanl.gov/.

Substitutions or insertions of an HIV Env, Gag, or Pol to obtain an additional HIV Env, Gag, or Pol, encoded by a nucleic acid for use in a recombinant MVA virus of the present invention, can include substitutions or insertions of at least one amino acid residue (e.g., 1-25 amino acids). Alternatively, at least one amino acid (e.g., 1-25 amino acids) can be deleted from an HIV Env, Gag, or Pol sequence. Preferably, such substitutions, insertions or deletions are identified based on safety features, expression levels, immunogenicity and compatibility with high replication rates of MVA.

Amino acid sequence variations in an HIV Env, Gag, or Pol of the present invention can be prepared e.g., by mutations in the DNA. Such HIV Env, Gag, or Pol include, for example, deletions, insertions or substitutions of nucleotides coding for different amino acid residues within the amino acid sequence. Obviously, mutations that will be made in nucleic acid encoding an HIV Env, Gag, or Pol must not place the sequence out of reading frame and preferably will not create complementary domains that could produce secondary mRNA structures.

HIV Env, Gag, or Pol-encoding nucleic acid of the present invention can also be prepared by amplification or site-directed mutagenesis of nucleotides in DNA or RNA encoding an HIV Env, Gag, or Pol and thereafter synthesizing or reverse transcribing the encoding DNA to produce DNA or RNA encoding an HIV Env, Gag, or Pol, based on the teaching and guidance presented herein.

Recombinant MVA viruses expressing HIV Env, Gag, or Pol of the present invention, include a finite set of HIV Env, Gag, or Pol-encoding sequences as substitution nucleotides that can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, 1978 *Principles of Protein Structure*, Springer-Verlag, New York, N.Y., and Creighton, T.E., 1983 *Proteins:*

Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, CA. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel *et al.* eds. *Current Protocols in Molecular Biology*, Greene Publishing Assoc., New York, N.Y. 1994 at §§ A.1.1-A.1.24, and Sambrook, J. *et al.* 1989 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. at Appendices C and D.

Thus, one of ordinary skill in the art, given the teachings and guidance presented herein, will know how to substitute other amino acid residues in other positions of an HIV *env*, *gag*, or *pol* DNA or RNA to obtain alternative HIV Env, Gag, or Pol, including substitutional, deletional or insertional variants.

Within the MVA vector, regulatory sequences for expression of the encoded antigen will include a natural, modified or synthetic poxvirus promoter. By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter. Other regulatory sequences including terminator fragments, polyadenylation sequences, marker genes and other sequences may be included as appropriate, in accordance with the knowledge and practice of the ordinary person skilled in the art: see, for example, Moss, B. (2001). Poxviridae: the viruses and their replication. In Fields Virology, D.M. Knipe, and P.M. Howley, eds. (Philadelphia, Lippincott Williams & Wilkins), pp. 2849-2883. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, 1998 Ausubel *et al.* eds., John Wiley & Sons.

Promoters for use in aspects and embodiments of the present invention must be compatible with poxvirus expression systems and include natural, modified and synthetic sequences.

Either or both of the priming and boosting compositions may include an adjuvant, such as granulocyte macrophage-colony stimulating factor (GM-CSF) or encoding nucleic acid therefor.

Administration of the boosting composition is generally about 1 to 6 months after administration of the priming composition, preferably about 1 to 3 months.

Preferably, administration of priming composition, boosting composition, or both priming and boosting compositions, is intradermal, intramuscular or mucosal immunization.

Administration of MVA vaccines may be achieved by using a needle to inject a suspension of the virus. An alternative is the use of a needleless injection device to administer a virus suspension (using, e.g., Biojector™ needleless injector) or a resuspended freeze-dried powder containing the vaccine, providing for manufacturing individually prepared doses that do not need cold storage. This would be a great advantage for a vaccine that is needed in rural areas of Africa.

MVA is a virus with an excellent safety record in human immunizations. The generation of recombinant viruses can be accomplished simply, and they can be manufactured reproducibly in large quantities. Intradermal, intramuscular or mucosal administration of recombinant MVA virus is therefore highly suitable for prophylactic or therapeutic vaccination of humans against AIDS which can be controlled by a $CD8^+$ T cell response.

The individual may have AIDS such that delivery of the antigen and generation of a $CD8^+$ T cell immune response to the antigen is of benefit or has a therapeutically beneficial effect.

Most likely, administration will have prophylactic aim to generate an immune response against HIV or AIDS before infection or development of symptoms.

Components to be administered in accordance with the present invention may be formulated in pharmaceutical compositions. These compositions may comprise a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

As noted, administration is preferably intradermal, intramuscular or mucosal.

Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous, subcutaneous, intramuscular or mucosal injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included as required.

A slow-release formulation may be employed.

Following production of MVA particles and optional formulation of such particles into compositions, the particles may be administered to an individual, particularly human or other primate. Administration may be to another mammal, e.g. rodent such as mouse, rat or hamster, guinea pig, rabbit, sheep, goat, pig, horse, cow, donkey, dog or cat.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, or in a veterinary context a veterinarian, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in *Remington's Pharmaceutical Sciences*, 16th edition, 1980, Osol, A. (ed.).

In one preferred regimen, DNA is administered at a dose of 250 μg to 2.5 mg/injection, followed by MVA at a dose of 10^6 to 10^9 infectious virus particles/injection.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Delivery to a non-human mammal need not be for a therapeutic purpose, but may be for use in an experimental context, for instance in investigation of mechanisms of immune responses to an antigen of interest, e.g. protection against HIV or AIDS.

Further aspects and embodiments of the present invention will be apparent to those of ordinary skill in the art, in view of the above disclosure and following experimental

exemplification, included by way of illustration and not limitation, and with reference to the attached figures.

EXAMPLE 1

Control of a Mucosal Challenge and Prevention of AIDS by a Multiprotein DNA/MVA Vaccine

Here we tested DNA priming and poxvirus boosting for the ability to protect against a highly pathogenic mucosal challenge. The 89.6 chimera of simian and human immunodeficiency viruses (SHIV-89.6) was used for the construction of immunogens and its highly pathogenic derivative, SHIV-89.6P, for challenge (G.B. Karlsson *et al.* 1997 *J Virol* **71**:4218). SHIV-89.6 and SHIV-89.6P do not generate cross-neutralizing antibody (D.C... Montefiori *et al.* 1998 *J Virol* **72**:3427) and allowed us to address the ability of vaccine-raised T cells and non-neutralizing antibodies to control an immunodeficiency virus challenge. Modified vaccinia Ankara (MVA) was used for the construction of the recombinant poxvirus. MVA has been highly effective at boosting DNA-primed CD8 T cells and enjoys the safety feature of not replicating efficiently in human or monkey cells (H.L. Robinson *et al.* 2000 *AIDS Reviews* **2**:105).

To ensure a broad immune response both the DNA and recombinant MVA (rMVA) components of the vaccine expressed multiple immunodeficiency virus proteins. The DNA prime (DNA/89.6) expressed simian immunodeficiency virus (SIV) Gag, Pol, Vif, Vpx, and Vpr and human immunodeficiency virus-1 (HIV-1) Env, Tat, and Rev from a single transcript (R.J. Gorelick *et al.* 1999 *Virology* **253**:259; M.M. Sauter *et al.* 1996 *J Cell Biol* **132**:795).

Molecularly cloned SHIV-89.6 sequences were cloned into the vector pGA2 using ClaI and RsrII sites. This cloning deleted both long terminal repeats (LTRs) and *nef*. The SHIV-89.6 sequences also were internally mutated for a 12-base pair region encoding the first four amino acids of the second zinc finger in nucleocapsid. This mutation renders SHIV viruses noninfectious (R.J. Gorelick *et al.* 1999 *Virology* **253**:259). A mutation in gp41 converted the tyrosine at position 710 to cysteine to achieve better expression of Env on the plasma membrane of DNA-expressing cells (M.M. Sauter *et al.* 1996 *J Cell Biol* **132**:795). pGA2 uses the CMV immediate early promoter without intron A and the bovine growth hormone polyadenylation sequence to express vaccine inserts. Vaccine DNA was

produced by Althea (San Diego, CA). In transient transfections of 293T cells, DNA/89.6 produced about 300 ng of Gag and 85 ng of Env per $1x10^6$ cells.

The rMVA booster (MVA/89.6) expressed SIV Gag, Pol, and HIV-1 Env under the control of vaccinia virus early/late promoters.

The MVA double recombinant virus expressed both the HIV 89.6 Env and the SIV 239 Gag-Pol, which were inserted into deletion II and deletion III of MVA, respectively. The 89.6 Env protein was truncated for the COOH-terminal 115 amino acids of gp41. The modified H5 promoter controlled the expression of both foreign genes.

Vaccination was accomplished by priming with DNA at 0 and 8 weeks and boosting with rMVA at 24 weeks (Fig. 1A).

I.d. and i.m. DNA immunizations were delivered in phosphate-buffered saline (PBS) with a needleless jet injector (Bioject, Portland, OR) to deliver five i.d. 100-µl injections to each outer thigh for the 2.5-mg dose of DNA or one i.d. 100-µl injection to the right outer thigh for the 250-µg dose of plasmid. I.m. deliveries of DNA were done with one 0.5-ml injection of DNA in PBS to each outer thigh for the 2.5-mg dose and one 100-µl injection to the right outer thigh for the 250-µg dose. $1x10^8$ pfu of MVA/89.6 was administered both i.d. and i.m. with a needle. One 100-µl dose was delivered to each outer thigh for the i.d. dose and one 500-µl dose to each outer thigh for the i.m dose. Control animals received 2.5 mg of the pGA2 vector without vaccine insert with the Bioject device to deliver five 100-µl doses i.d. to each outer thigh. The control MVA booster immunization consisted of $2x10^8$ pfu of MVA without an insert delivered i.d. and i.m. as described for MVA/89.6.

Four groups of six rhesus macaques each were primed with either 2.5 mg (high-dose) or 250 µg (low-dose) of DNA by intradermal (i.d.) or intramuscular (i.m.) routes using a needleless jet injection device (Bioject, Portland, OR) (T.M. Allen *et al.* 2000 *J Immunol* **164**:4968).

Young adult rhesus macaques from the Yerkes breeding colony were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" with protocols approved by the Emory University Institutional Animal Care and Use Committee. Macaques were typed for the *Mamu-A*01* allele with polymerase chain reaction (PCR) analyses (M.A. Egan *et al.* 2000 *J Virol* **74**:7485; I. Ourmanov *et al.* 2000 *J Virol* **74**:2740). Two or more animals containing at

least one *Mamu-A*01* allele were assigned to each group. Animal numbers are as follows: 1, RBr-5*; 2, RIm-5*; 3, RQf-5*; 4, RZe-5; 5, ROm-5; 6, RDm-5; 7, RAj-5*; 8, RJi-5*; 9, RAl-5*; 10, RDe-5*; 11, RAi-5; 12, RPr-5; 13, RKw-4*; 14, RWz-5*; 15, RGo-5; 16, RLp-4; 17, RWd-6; 18, RAt-5; 19, RPb-5*; 20, RIi-5*; 21, RIq-5; 22, RSp-4; 23, RSn-5; 24, RGd-6; 25, RMb-5*; 26, RGy-5*; 27, RUs-4; and 28, RPm-5. Animals with the *A*01* allele are indicated with asterisks.

Gene gun deliveries of DNA were not used because these had primed non-protective immune responses in a 1996 - 98 trial (H.L. Robinson *et al.* 1999 *Nat Med* 5:526). The MVA/89.6 booster immunization ($2x10^8$ plaque-forming units, pfu) was injected with a needle both i.d. and i.m. A control group included two mock immunized animals and two naive animals. The challenge was given at 7 months after the rMVA booster to test for the generation of long-term immunity. Because most HIV-1 infections are transmitted across mucosal surfaces, an intrarectal challenge was administered.

DNA priming followed by rMVA boosting generated high frequencies of virus-specific T cells that peaked at one week following the rMVA booster (Fig. 1). The frequencies of T cells recognizing the Gag-CM9 epitope were assessed by means of Mamu-A*01 tetramers, and the frequencies of T cells recognizing epitopes throughout Gag were assessed with pools of overlapping peptides and an enzyme-linked immunospot (ELISPOT) assay (C.A. Power *et al.* 1999 *J Immunol Methods* **227**:99).

For tetramer analyses, about $1x10^6$ peripheral blood mononuclear cells (PBMC) were surface-stained with antibodies to CD3 conjugated to fluorescein isothiocyanate (FITC) (FN-18; Biosource International, Camarillo, CA), CD8 conjugated to peridinin chlorophyl protein (PerCP) (SK1; Becton Dickinson, San Jose, CA), and Gag-CM9 (CTPYDINQM)-*Mamu-A*01* tetramer (SEQ ID NO: 6) conjugated to allophycocyanin (APC), in a volume of 100 µl at 8° to 10°C for 30 min. Cells were washed twice with cold PBS containing 2% fetal bovine serum (FBS), fixed with 1% paraformaldehyde in PBS, and analyzed within 24 hrs on a FACScaliber (Becton Dickinson, San Jose, CA). Cells were initially gated on lymphocyte populations with forward scatter and side scatter and then on CD3 cells. The CD3 cells were then analyzed for CD8 and tetramer-binding cells. About 150,000 lymphocytes were acquired for each sample. Data were analyzed using FloJo software (Tree Star, San Carlos, CA).

For interferon-γ (IFN-γ) ELISPOTs, MULTISCREEN 96 well filtration plates (Millipore Inc. Bedford, MA) were coated overnight with antibody to human IFN-γ (Clone B27, Pharmingen, San Diego, CA) at a concentration of 2 μg/ml in sodium bicarbonate buffer (pH 9.6) at 8° to 10°C. Plates were washed two times with RPMI medium and then blocked for 1 hour with complete medium (RPMI containing 10% FBS) at 37°C. Plates were washed five more times with plain RPMI medium, and cells were seeded in duplicate in 100 μl complete medium at numbers ranging from $2x10^4$ to $5x10^5$ cells per well. Peptide pools were added to each well to a final concentration of 2 μg/ml of each peptide in a volume of 100 μl in complete medium. Cells were cultured at 37°C for about 36 hrs under 5% CO_2. Plates were washed six times with wash buffer (PBS with 0.05% Tween-20) and then incubated with 1 μg of biotinylated antibody to human IFN-γ per milliliter (clone 7-86-1; Diapharma Group, West Chester, OH) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 hrs at 37°C and washed six times with wash buffer. Avidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) was added to each well and incubated for 30 to 60 min at 37°C. Plates were washed six times with wash buffer and spots were developed using stable DAB as substrate (Research Genetics, Huntsville, AL). Spots were counted with a stereo dissecting microscope. An ovalbumin peptide (SIINFEKL) (SEQ ID NO: 7) was included as a control in each analysis. Background spots for the ovalbumin peptide were generally <5 for $5x10^5$ PBMCs. This background when normalized for $1x10^6$ PBMC was <10. Only ELISPOT counts of twice the background (≥20) were considered significant. The frequencies of ELISPOTs are approximate because different dilutions of cells have different efficiencies of spot formation in the absence of feeder cells (C.A. Power *et al.* 1999 *J Immunol Methods* **227**: 99). The same dilution of cells was used for all animals at a given time point, but different dilutions were used to detect memory and acute responses.

Gag-CM9 tetramer analyses were restricted to macaques that expressed the *Mamu-A*01* histocompatibility type, whereas ELISPOT responses did not depend on a specific histocompatibility type. As expected, the DNA immunizations raised low levels of memory cells that expanded to high frequencies within 1 week of the rMVA booster (Fig. 1 and 6). In *Mamu-A*01* macaques, CD8 cells specific to the Gag-CM9 epitope expanded to frequencies as high as 19% of total CD8 T cells (Fig. 6). This peak of specific cells underwent a 10- to 100-fold contraction into the DNA/MVA memory pool (Fig. 1A and 6).

ELISPOTs for three pools of Gag peptides also underwent a major expansion (frequencies up to 4000 spots for $1x10^6$ PBMC) before contracting from 5- to 20-fold into the DNA/MVA memory response (Fig. 1B). The frequencies of ELISPOTs were the same in macaques with and without the *A*01* histocompatibility type (P>0.2).

Simple linear regression was used to estimate correlations between postbooster and postchallenge ELISPOT responses, between memory and postchallenge ELISPOT responses, and between logarithmically transformed viral loads and ELISPOT frequencies. Comparisons between vaccine and control groups and *A*01* and non *A*01* macaques were performed by means of two-sample *t* tests with logarithmically transformed viral load and ELISPOT responses. Two-way analyses of variance were used to examine the effects of dose and route of administration on peak DNA/MVA ELISPOTs, on memory DNA/MVA ELISPOTs, and on logarithmically transformed Gag antibody data.

At both peak and memory phases of the vaccine response, the rank order for the height of the ELISPOTs in the vaccine groups was 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m. (Fig. 1B). The IFN-γ ELISPOTs included both CD4 and CD8 cells. Gag-CM9-specific CD8 cells had good lytic activity after restimulation with peptide.

The highly pathogenic SHIV-89.6P challenge was administered intrarectally at 7 months after the rMVA booster, when vaccine-raised T cells were in memory (Fig. 1).

The challenge stock (5.7 x 10^9 copies of viral RNA per milliliter) was produced by one intravenous followed by one intrarectal passage in rhesus macaques of the original SHIV-89.6P stock (G.B. Karlsson *et al.* 1997 *J Virol* **71**:4218). Lymphoid cells were harvested from the intrarectally infected animal at peak viremia, CD8-depleted, and mitogen-stimulated for stock production. Before intrarectal challenge, fasted animals were anesthetized (ketamine, 10 mg/kg) and placed on their stomach with the pelvic region slightly elevated. A feeding tube (8Fr (2.7 mm) x 16 inches (41 cm); Sherwood Medical, St. Louis, MO) was inserted into the rectum for a distance of 15 to 20 cm. Following insertion of the feeding tube, a syringe containing 20 intrarectal infectious doses in 2 ml of RPMI-1640 plus 10% FBS was attached to the tube and the inoculum was slowly injected into the rectum. After delivery of the inoculum, the feeding tube was flushed with 3.0 ml of RPMI without FBS and then slowly withdrawn. Animals were left in place, with pelvic regions slightly elevated, for a period of ten minutes after the challenge.

The challenge infected all of the vaccinated and control animals (Fig. 2). However, by 2 weeks after challenge, titers of plasma viral RNA were at least 10-fold lower in the vaccine groups (geometric means of $1x10^7$ to $5x10^7$) than in the control animals (geometric mean of $4x10^8$) (Fig. 2A) (S. Staprans *et al.* in: *Viral Genome Methods* K. Adolph, ed. CRC Press, Boca Raton, FL, 1996 pp. 167-184; R. Hofmann-Lehmann *et al.* 2000 *AIDS Res Hum Retroviruses* **16**:1247).

For the determination of SHIV copy number, viral RNA from 150 µl of ACD anticoagulated plasma was directly extracted with the QIAamp Viral RNA kit (Qiagen), eluted in 60 µl of AVE buffer, and frozen at -80°C until SHIV RNA quantitation was performed. Five microliters of purified plasma RNA was reverse-transcribed in a final 20-µl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate (dNTP), 2.5 µM random hexamers, 20 units MultiScribe RT, and 8 units ribonuclease inhibitor. Reactions were incubated at 25°C for 10 min, followed by incubation at 42°C for 20 min, and inactivation of reverse transcriptase at 99°C for 5 min. The reaction mix was adjusted to a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.1 µM probe, and 5 units AmpliTaq Gold DNA polymerase (all reagents from PerkinElmer Applied Biosystems, Foster City, CA). The primer sequences within a conserved portion of the SIV *gag* gene are the same as those described previously (S. Staprans *et al.* in: *Viral Genome Methods* K. Adolph, ed. CRC Press, Boca Raton, FL, 1996 pp. 167-184). A PerkinElmer Applied Biosystems 7700 Sequence Detection System was used with the PCR profile: 95°C for 10 min, followed by 40 cycles at 93°C for 30 s, and 59.5°C for 1 min. PCR product accumulation was monitored with the 7700 sequence detector and a probe to an internal conserved *gag* gene sequence: 6FAM-CTGTCTGCGTCATTTGGTGC-Tamra (SEQ ID NO: 8), where FAM and Tamra denote the reporter and quencher dyes. SHIV RNA copy number was determined by comparison with an external standard curve consisting of virion-derived SIVmac239 RNA quantified by the SIV bDNA method (Bayer Diagnostics, Emeryville, CA). All specimens were extracted and amplified in duplicate, with the mean result reported. With a 0.15-ml plasma input, the assay has a sensitivity of 10^3 RNA copies per milliliter of plasma and a linear dynamic range of 10^3 to 10^8 RNA copies (R^2 = 0.995). The intraassay coefficient of variation was <20% for samples containing $>10^4$ SHIV RNA copies per milliliter, and

<25% for samples containing 10^3 to 10^4 SHIV RNA copies per milliliter. To more accurately quantitate low SHIV RNA copy number in vaccinated animals at weeks 16 and 20, we made the following modifications to increase the sensitivity of the SHIV RNA assay: (i) Virions from ≤1 ml of plasma were concentrated by centrifugation at 23,000g at 10°C for 150 min before viral RNA extraction, and (ii) a one-step reverse transcriptase PCR method was used (R. Hofmann-Lehmann *et al.* 2000 *AIDS Res Hum Retroviruses* **16**:1247). These changes provided a reliable quantification limit of 300 SHIV RNA copies per milliliter, and gave SHIV RNA values that were highly correlated to those obtained by the first method used ($r = 0.91$, $P<0.0001$).

By 8 weeks after challenge, both high-dose DNA-primed groups and the low-dose i.d. DNA-primed group had reduced their geometric mean loads to about 1000 copies of viral RNA per milliliter. At this time, the low-dose i.m. DNA-primed group had a geometric mean of $6x10^3$ copies of viral RNA and the nonvaccinated controls had a geometric mean of 2×10^6. By 20 weeks after challenge, even the low-dose i.m. group had reduced its geometric mean copies of viral RNA to 1000. Among the 24 vaccinated animals, only one animal, animal number 22 in the low-dose i.m. group, had intermittent viral loads above $1x10^4$ copies per milliliter (Fig 2D).

By 5 weeks after challenge, all of the nonvaccinated controls had undergone a profound depletion of CD4 cells (Fig 2B). All of the vaccinated animals maintained their CD4 cells, with the exception of animal 22 in the low dose i.m. group (see above), which underwent a slow CD4 decline (Fig. 2E). By 23 weeks after challenge, three of the four control animals had succumbed to AIDS (Fig. 2C). These animals had variable degrees of enterocolitis with diarrhea, cryptosporidiosis, colicystitis, enteric campylobacter infection, splenomegaly, lymphadenopathy, and SIV-associated giant cell pneumonia. In contrast, all 24 vaccinated animals maintained their health.

Containment of the viral challenge was associated with a burst of antiviral T cells (Fig. 1 and 3A). At one week after challenge, the frequency of tetramer$^+$ cells in the peripheral blood had decreased, potentially reflecting the recruitment of specific T cells to the site of infection (Fig. 3A). However, by two weeks after challenge, tetramer$^+$ cells in the peripheral blood had expanded to frequencies as high as, or higher than, after the rMVA booster (Fig. 1 and 3A). The majority of the tetramer$^+$ cells produced IFN-γ in response to a 6-hour peptide stimulation (Fig. 3B) (S.L. Waldrop *et al.* 1997 *J Clin Invest* **99**:1739) and

did not have the "stunned" IFN-γ negative phenotype sometimes observed in viral infections (F. Lechner *et al.* 2000 *J Exp Med* **191**:1499).

For intracellular cytokine assays, about $1x10^6$ PBMC were stimulated for 1 hour at 37°C in 5 ml polypropylene tubes with 100 μg of Gag-CM9 peptide (CTPYDINQM) (SEQ ID NO: 6) per milliliter in a volume of 100 μl RPMI containing 0.1% bovine serum albumin (BSA) and 1 μg of antibody to human CD28 and 1 μg of antibody to human CD49d (Pharmingen, San Diego, CA) per milliliter. Then, 900 μl of RPMI containing 10% FBS and monensin (10 μg/ml) was added, and the cells were cultured for an additional 5 hrs at 37°C at an angle of 5° under 5% CO_2. Cells were surface stained with antibodies to CD8 conjugated to PerCP (clone SK1, Becton Dickinson) at 8° to 10°C for 30 min, washed twice with cold PBS containing 2% FBS, and fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen). Cells were then incubated with antibodies to human CD3 (clone FN-18; Biosource International, Camarillo, CA) and IFN-γ (Clone B27; Pharmingen) conjugated to FITC and phycoerythrin, respectively, in Perm wash solution (Pharmingen) for 30 min at 4°C. Cells were washed twice with Perm wash, once with plain PBS, and resuspended in 1% paraformaldehyde in PBS. About 150,000 lymphocytes were acquired on the FACScaliber and analyzed with FloJo software.

The postchallenge burst of T cells contracted concomitant with the decline of the viral load. By 12 weeks after challenge, virus-specific T cells were present at about one-tenth of their peak height (Figs. 1A and 3A). In contrast to the vigorous secondary response in the vaccinated animals, the naive animals mounted a modest primary response (Fig. 1B and 3A). Tetramer$^+$ cells peaked at less than 1% of total CD8 cells (Fig. 3A), and IFN-γ-producing ELISPOTs were present at a mean frequency of about 300 as opposed to the much higher frequencies of 1000 to 6000 in the vaccine groups (Fig. 1B) ($P<0.05$).

The tetramer$^+$ cells in the control group, like those in the vaccine group, produced IFN-γ after peptide stimulation (Fig. 3B). By 12 weeks after challenge, three of the four controls had undetectable levels of IFN-γ-producing ELISPOTs. This rapid loss of antiviral T cells in the presence of high viral loads may reflect the lack of CD4 help.

T cell proliferative responses demonstrated that virus-specific CD4 cells had survived the challenge and were available to support the antiviral immune response (Fig. 3C).

About 0.2 million PBMC were stimulated in triplicate for 5 days with the indicated antigen in 200 µl of RPMI at 37°C under 5% CO_2. Supernatants from 293T cells transfected with DNA expressing either SHIV-89.6 Gag and Pol or SHIV-89.6 Gag, Pol and Env were used directly as antigens (final concentration of ~0.5 µg of p27 Gag per milliliter). Supernatants from mock DNA (vector alone)-transfected cells served as negative controls. On day six, cells were pulsed with 1 µCi of tritiated thymidine per well for 16 to 20 hours. Cells were harvested with an automated cell harvester (TOMTEC, Harvester 96, Model 1010, Hamden, CT) and counted with a Wallac 1450 MICROBETA Scintillation counter (Gaithersburg, MD). Stimulation indices are the counts of tritiated thymidine incorporated in PBMC stimulated with 89.6 antigens divided by the counts of tritiated thymidine incorporated by the same PBMC stimulated with mock antigen.

At 12 weeks after challenge, mean stimulation indices for Gag-Pol-Env or Gag-Pol proteins ranged from 35 to 14 in the vaccine groups but were undetectable in the control group. Consistent with the proliferation assays, intracellular cytokine assays demonstrated the presence of virus-specific CD4 cells in vaccinated but not control animals. The overall rank order of the vaccine groups for the magnitude of the proliferative response was 2.5 mg i.d. > 2.5 mg i.m. > 250 µg i.d. > 250 µg i.m.

At 12 weeks after challenge, lymph nodes from the vaccinated animals were morphologically intact and responding to the infection, whereas those from the infected controls had been functionally destroyed (Fig. 4). Nodes from vaccinated animals contained large numbers of reactive secondary follicles with expanded germinal centers and discrete dark and light zones (Fig. 4A). By contrast, lymph nodes from the non-vaccinated control animals showed follicular and paracortical depletion (Fig. 4B), while those from unvaccinated and unchallenged animals displayed normal numbers of minimally reactive germinal centers (Fig. 4C). Germinal centers occupied < 0.05% of total lymph node area in the infected controls, 2% of the lymph node area in the uninfected controls, and up to 18% of the lymph node area in the vaccinated groups (Fig. 4D). More vigorous immune reactivity in the low-dose than the high-dose DNA-primed animals was suggested by more extensive germinal centers in the low dose group (Fig. 4D). At 12 weeks after challenge, *in situ* hybridization for viral RNA revealed rare virus-expressing cells in lymph nodes from 3 of the 24 vaccinated macaques, whereas virus-expressing cells were readily detected in lymph nodes from each of the infected control animals. In the controls, which had

undergone a profound depletion in CD4 T cells, the cytomorphology of infected lymph node cells was consistent with a macrophage phenotype.

The prime/boost strategy raised low levels of antibody to Gag and undetectable levels of antibody to Env (Fig. 5). Postchallenge, antibodies to both Env and Gag underwent anamnestic responses with total Gag antibody reaching heights approaching 1 mg/ml and total Env antibody reaching heights of up to 100 μg/ml.

Enzyme-linked immunosorbent assays (ELISAs) for total antibody to Gag used bacterially produced SIV gag p27 to coat wells (2 μg per milliliter in bicarbonate buffer). ELISAs for antibody to Env antibody used 89.6 Env produced in transiently transfected 293T cells and captured with sheep antibody against Env (catalog number 6205; International Enzymes, Fairbrook CA). Standard curves for Gag and Env ELISAs were produced with serum from a SHIV-89.6-infected macaque with known amounts of immunoglobulin G (IgG) specific for Gag or Env. Bound antibody was detected with peroxidase-conjugated goat antibody to macaque IgG (catalog # YNGMOIGGFCP; Accurate Chemical, Westbury, NY) and TMB substrate (Catalog # T3405; Sigma, St. Louis, MO). Sera were assayed at threefold dilutions in duplicate wells. Dilutions of test sera were performed in whey buffer (4% whey and 0.1% tween 20 in 1X PBS). Blocking buffer consisted of whey buffer plus 0.5% nonfat dry milk. Reactions were stopped with 2M H_2SO_4 and the optical density read at 450 nm. Standard curves were fitted and sample concentrations were interpolated as μg of antibody per ml of serum using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA).

By 2 weeks after challenge, neutralizing antibodies for the 89.6 immunogen, but not the SHIV-89.6P challenge, were present in the high-dose DNA-primed groups (geometric mean titers of 352 in the i.d. and 303 in the i.m. groups) (Fig. 5C) (D.C. Montefiori *et al.* 1988 *J Clin* Microbiol **26**:231). By 5 weeks after challenge, neutralizing antibody to 89.6P had been generated (geometric mean titers of 200 in the high-dose i.d. and 126 in the high-dose i.m. group) (Fig. 5D) and neutralizing antibody to 89.6 had started to decline. By 16 to 20 weeks after challenge, antibodies to Gag and Env had fallen in most animals.

Our results demonstrate that a multiprotein DNA/MVA vaccine can raise a memory immune response capable of controlling a highly virulent mucosal immunodeficiency virus challenge. Our levels of viral control were more favorable than have been achieved using only DNA (M.A. Egan *et al.* 2000 *J Virol* **74**:7485) or rMVA vaccines (I. Ourmanov *et al.*

2000 *J Virol* **74**:2740) and were comparable to those obtained for DNA immunizations adjuvanted with interleukin-2 (D.H. Barouch *et al.* 2000 *Science* **290**:486). All of these previous studies have used more than three vaccine inoculations, none have used mucosal challenges, and most have challenged at peak effector responses and not allowed a prolonged post vaccination period to test for "long term" efficacy.

The dose of DNA had statistically significant effects on both cellular and humoral responses ($P<0.05$), whereas the route of DNA administration affected only humoral responses. Intradermal DNA delivery was about 10 times more effective than i.m. inoculations for generating antibody to Gag ($P = 0.02$). Neither route nor dose of DNA appeared to have a significant effect on protection. At 20 weeks after challenge, the high-dose DNA-primed animals had slightly lower geometric mean levels of viral RNA ($7x10^2$ and $5x10^2$) than the low-dose DNA-primed animals ($9x10^2$ and $1x10^3$).

The DNA/MVA vaccine controlled the infection, rapidly reducing viral loads to near or below 1000 copies of viral RNA per milliliter of blood. Containment, rather than prevention of infection, affords the opportunity to establish a chronic infection (H.L. Robinson *et al.* 1999 *Nat Med* **5**:526). By rapidly reducing viral loads, a multiprotein DNA/MVA vaccine will extend the prospect for long-term non-progression and limit HIV transmission. (J.W. Mellors *et al.* 1996 *Science* **272**:1167; T.C. Quinn *et al.* 2000 *N Engl J Med* **342**:921).

EXAMPLE 2

MVA Expressing Modified HIV Env, Gag, and Pol Genes

This disclosure describes the construction of a modified vaccinia Ankara (MVA) recombinant virus, MVA/HIV clade B recombinant virus expressing the HIV strain ADA env and the HXB2 gag pol (MVA/HIV ADA env + HXB2 gag pol). For amplification, the lab name of MVA/HIV 48 will be used, which denotes the plasmid from which the construct comes.

The HIV *gag-pol* genes were derived from the Clade B infectious HXB2 virus. The *gag-pol* gene was truncated so that most of the integrase coding sequences were removed and amino acids 185, 266, and 478 were mutated to inactivate reverse transcriptase, inhibit strand transfer activity, and inhibit the RNaseH activity, respectively. The Clade B CCR5 tropic envelope gene was derived from the primary ADA isolate; TTTTTNT sequences were mutated without changing coding capacity to prevent premature transcription

termination and the cytoplasmic tail was truncated in order to improve surface expression, immunogenicity, and stability of the MVA vector. The HIV genes were inserted into a plasmid transfer vector so that *gag-pol* gene was regulated by the modified H5 early/late vaccinia virus promoter and the *env* gene was regulated by the newly designed early/late Psyn II promoter to provide similar high levels of expression. A self-deleting GUS reporter gene was included to allow detection and isolation of the recombinant virus. The HIV genes were flanked by MVA sequences to allow homologous recombination into the deletion 3 site so that the recombinant MVA would remain TK positive for stability and high expression in resting cells. The recombinant MVA was isolated and shown to express abundant amounts of *gag-pol-env* and to process *gag*. Production of HIV-like particles was demonstrated by centrifugation and by electron microscopy. The presence of env in the HIV-like particles was demonstrated by immunoelectron microscopy.

Table of Sequences

Description	SEQ ID NO	FIG. NO
pLW-48	1	A
pLW-48	1	B
Psyn II promoter	2	B
ADA envelope truncated	3	B
PmH5 promoter	4	B
HXB2 *gag pol*	5	B

Plasmid Transfer Vector

The plasmid transfer vector used to make the MVA recombinant virus, pLW-48, (Figure C) by homologous recombination was constructed as follows:

1. From the commercially obtained plasmid, pGem-4Z (Promega), flanking areas on either side of deletion III, designated flank 1 and flank 2, containing 926 and 520 base pairs respectively, were amplified by PCR from the MVA stains of vaccinia virus. Within these flanks, a promoter, the mH5, which had been modified from the originally published sequence by changing two bases that had been shown by previously published work to increase the expression of the cloned gene, was added.

2. A clade B gag pol (Figure D) was truncated so that the integrase was removed and was cloned into the plasmid so that it was controlled by the mH5 promoter. This gene contained the complete HXB2 sequence of the gag. The pol gene has reverse transcriptase safety mutations in amino acid 185 within the active site of RT, in amino acid 266 which inhibits strand transfer activity, and at amino acid 478 which inhibits the RNaseH activity. In addition, the integrase gene was deleted past EcoRI site.

3. A direct repeat of 280 basepairs, corresponding to the last 280 base pairs of MVA flank 1, was added after flank 1.

4. The p11 promoter and GUS reporter gene were added between the two direct repeats of flank 1 so that this screening marker could initially be used for obtaining the recombinant virus, yet deleted out in the final recombinant virus (Scheiflinger, F. *et al.* 1998 *Arch Virol* **143**:467-474; Carroll, M.W. and B. Moss 1995 *BioTechniques* **19**:352-355).

5. A new promoter, Psyn II, was designed to allow for increased expression of the ADA env. The sequence of this new early/late promoter is given in Figure E.

6. A truncated version of the ADA envelope with a silent 5TNT mutation was obtained by PCR and inserted in the plasmid under the control of the Psyn II promoter. The envelope was truncated in the cytoplasmic tail of the gp41 gene, deleting 115 amino acids of the cytoplasmic tail. This truncation was shown to increase the amount of envelope protein on the surface of infected cells and enhance immunogenicity of the envelope protein in mice, and stability of the recombinant virus in tissue culture.

Recombinant MVA Construction

1. MVA virus, which may be obtained from ATCC Number VR-1508, was plaque purified three times by terminal dilutions in chicken embryo fibroblasts (CEF),which were made from 9 day old SPF Premium SPAFAS fertile chicken eggs, distributed by B and E Eggs, Stevens, PA.

2. Secondary CEF cells were infected at an MOI of 0.05 of MVA and transfected with 2 μg of pLW-48, the plasmid described above. Following a two day incubation at 37°C, the virus was harvested, frozen and thawed 3x, and plated out on CEF plates.

3. At 4 days, those foci of infection that stained blue after addition of X-gluc substrate, indicating that recombination had occurred between the plasmid and the infecting

virus, were picked and inoculated on CEF plates. Again, those foci that stained blue were picked.

4. These GUS containing foci were plated out in triplicate and analyzed for GUS staining (which we wanted to now delete) and ADA envelope expression. Individual foci were picked from the 3rd replicate plates of those samples that had about equal numbers of mixed populations of GUS staining and nonstaining foci as well as mostly envelope staining foci.

5. These foci were again plated out in triplicate, and analyzed the same way. After 5 passages, a virus was derived which expressed the envelope protein but which had deleted the GUS gene because of the double repeat. By immunostaining, this virus also expressed the gag pol protein.

Characterization of MVA Recombinant Virus, MVA/HIV 48

1. Aliquots of MVA/HIV 48 infected cell lysates were analyzed by radioimmunoprecipitation and immunostaining with monoclonal antibodies for expression of both the envelope and gag pol protein. In both of these tests, each of these proteins was detected.

2. The recombinant virus was shown to produce gag particles in the supernatant of infected cells by pelleting the ^{35}S-labeled particles on a 20% sucrose cushion.

3. Gag particles were also visualized both outside and budding from cells as well as within vacuoles of cells in the electron microscope in thin sections. These gag particles had envelope protein on their surface.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer, and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the

actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Summary

In summary, we have made a recombinant MVA virus, MVA/HIV 48, which has high expression of the ADA truncated envelope and the HXB2 gag pol. The MVA recombinant virus is made using a transiently expressed GUS marker that is deleted in the final virus. High expression of the ADA envelope is possible because of a new hybrid early/late promoter, Psyn II. In addition, the envelope has been truncated because we have shown truncation of the envelope enhances the amount of protein on the surface of the infected cells, and hence enhances immunogenicity; stability of the recombinant is also enhanced. The MVA recombinant makes gag particles which has been shown by pelleting the particles through sucrose and analyzing by PAGE. Gag particles with envelope protein on the surface have also been visualized in the electron microscope.

EXAMPLE 3

Additional Modified or Synthetic Promoters Designed for Gene Expression in MVA Or Other Poxviruses

Additional modified or synthetic promoters were designed for gene expression in MVA or other poxviruses. Promoters were modified to allow expression at early and late times after infection and to reduce possibility of homologous recombination between identical sequences when multiple promoters are used in same MVA vector. Promoters are placed upstream of protein coding sequence.

m7.5 promoter (SEQ ID NO:10):

CGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCT
CGTAAAAATTGAAAAACTATTCTAATTTATTGCACGGT

Psyn II promoter (SEQ ID NO:2):

TAAAAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATGCTATAAATAATAAATA

Psyn III promoter (SEQ ID NO:11):

TAAAAATTGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATACTATAAATAATAAATA

Psyn IV promoter (SEQ ID NO:12):

TAAAAATTGAAAAACTATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATACTATAAATAATAAATA

PsynV promoter (SEQ ID NO:13):

AAAAAATGATAAAGTAGGTTCAGTTTTATTGCTGGTTTAAAATCACGCTTTCGA
GTAAAAACTACGAATATAAAT

EXAMPLE 4

Tables A-F

TableA: MVA/48 immunization – guinea pigs.

Groups of guinea pigs were immunized at days 0 and 30 with 1 $x10^8$ infectious units of MVA/48 by either the intramuscular (IM) or intradermal (ID) route. As a control another group was immunized IM with the same dose of non-recombinant MVA. Sera taken before as well as after each immunization was analyzed for neutralizing activity against HIV-1-MN. Titers are the reciprocal serum dilution at which 50% of MT-2 cells were protected from virus-induced killing. Significant neutralizing activity was observed in all animals after the second immunization with MVA/48 (day 49).

Table B: Frequencies of HIV-1 gag-specific T cells following immunization of mice with MVA/48.

Groups of BalbC mice were immunized at days 0 and 21 with 1 $x10^7$ infectious units of MVA/48 by one of three routes: intraperitoneal (IP), intradermal (ID), or intramuscular (IM). A control group was immunized with non-recombinant MVA. At 5 weeks after the last immunization, splenocytes were prepared and stimulated *in vitro* with an immunodominant peptide from HIV-1 p24 for 7 days. The cells were then mixed either with peptide-pulsed P815 cells or with soluble peptide. Gamma interferon-producing cells were enumerated in an ELISPOT assay. A value of >500 was assigned to wells containing too many spots to count. Strong T cell responses have been reported in mice immunized IP

with other viruses. In this experiment, IP immunization of mice with MVA/48 elicited very strong HIV-1 gag-specific T cell responses.

Table C: DNA prime and MVA/48 boost – total ELISPOTS per animal.

Ten rhesus macaques were primed (weeks 0 and 8) with a DNA vaccine expressing HIV-1 antigens including Ada envelope and HXB2 gagpol. At week 24 the animals were boosted intramuscularly with 1 $x10^8$ infectious units of MVA/48. Fresh peripheral blood mononuclear cells (PBMC) were analyzed for production of gamma interferon in an ELISPOT assay as follows: PBMC were incubated for 30-36 hours in the presence of pools of overlapping peptides corresponding to the individual HIV-1 antigens in the vaccines. The total number of gamma interferon-producing cells from each animal is shown in the table. T cell responses to DNA vaccination were limited (weeks 2-20). However, boosting with MVA/48 resulted in very strong HIV-1-specific T cell responses in all animals (week 25).

Table D: Antibody response following immunization of macaques with MVA/SHIV KB9.

Groups of rhesus macaques were immunized with 2 $x10^8$ infectious units of MVA/SHIV-KB9 at weeks 0 and 4 by one of several routes: Tonsilar, intradermal (ID), or intramuscular (IM). Another group was immunized with non-recombinant MVA using the same routes. Serum samples from 2 weeks after the second immunization were analyzed for binding to KB9 envelope protein by ELISA and for neutralization of SHIV-89.6P and SHIV-89.6. In the ELISA assay, soluble KB9 envelope protein was captured in 96 well plates using an antibody to the C-terminus of gp120. Serial dilutions of sera were analyzed and used to determine the endpoint titers. Neutralization of SHIV-89.6P and SHIV-89.6 was determined in an MT-2 cell assay. Titers are the reciprocal serum dilution at which 50% of the cells were protected from virus-induced killing. In *in vitro* neutralization assays, SHIV-89.6P and SHIV-89.6 are heterologous, i.e. sera from animals infected with one of the viruses does not neutralize the other virus. Thus, two immunizations with MVA/SHIV-KB9 elicited good ELISA binding antibodies in all animals and neutralizing antibodies to the homologous virus (SHIV-89.6P) in some animals. In addition, heterologous neutralizing antibodies were observed in a subset of animals.

Table E: Frequencies of gag CM-9-specific CD3/CD8 T cells following immunization of macaques with MVA/SHIV-KB9.

Groups of MamuA*01 positive rhesus macaques were immunized with 2 $x10^8$ infectious units of MVA/SHIV-KB9 at weeks 0 and 4 by one of several routes: tonsilar, intradermal (ID), or intramuscular IM). Another group was immunized with non-recombinant MVA. The frequencies of CD3+/CD8+ T cells that bound tetrameric complex containing the SIV gag-specific peptide CM9 were determined by flow cytometry at various times after each immunization. Time intervals were as follows: 1a, 1b, and 1d were one, two, and four weeks after the first immunization, respectively; 2a, 2b, 2c, and 2d were one, two, three, and twelve weeks after the second immunization, respectively. Values above background are shown in bold face. Strong SIV gag-specific responses were observed after a single immunization with MVA/SHIV-KB9 in all immunized animals. Boosting was observed in most animals following the second immunization. In addition, measurable tetramer binding was still found twelve weeks after the second immunization.

Table F: Frequencies of specific T cells following immunization of macaques with MVA/SHIV KB9.

Groups of macaques were immunized with MVA/SHIV-KB9 as described above. MVA/SHIV-KB9 expresses 5 genes from the chimeric virus, SHIV-89.6P: envelope, gag, polymerase, tat, and nef. Thus, the frequencies of T cells specific for each of the 5 antigens was analyzed using pools of peptides corresponding to each individual protein. Fresh PBMC were stimulated with pools of peptides for 30-36 hours in vitro. Gamma interferon-producing cells were enumerated in an ELISPOT assay. The total number of cells specific for each antigen is given as "total # spots". In addition, the number of responding animals and average # of spots per group is shown. PBMC were analyzed at one week after the first immunization (1a) and one week after the second immunization (2a). Another group of 7 animals was immunized with non-recombinant MVA. In these animals, no spots above background levels were detected. Thus, a single immunization with MVA/SHIV-KB9 elicited strong SHIV-specific T cell responses in all animals. Gag and envelope responses were the strongest; most animals had responses to gag, all animals had responses to envelope. The Elispot responses were also observed after the second immunization with MVA/SHIV-KB9, albeit at lower levels. At both times, the rank order of responses was: tonsilar > ID > IM. We show good immune response to nef and some immune response to tat.

TABLE A

MVA/48 immunization – guinea pigs
HIV-MN neutralizing antibody - reciprocal titer

Animal #	Group	Route	day 0	Day 4 MVA #1	day 30	day 33 MVA#2	day 49
885	MVA	I.M.	<20	I.M.	31	I.M.	24
891	"	"	<20	"	85	"	<20
882	MVA/48	I.M.	<20	I.M.	<20	I.M.	5,524
883	"	"	<20	"	68	"	691
886	"	"	<20	"	<20	"	4,249
890	"	"	<20	"	180	"	89
879	MVA/48	I.D.	<20	I.D.	<20	I.D.	817
881	"	"	<20	"	<20	"	234
888	"	"	<20	"	24	"	112
889	"	"	<20	"	22	"	376

TABLE B

Frequencies of HIV-gag-specific T cells following immunization of mice with MVA/48

Group	P815 cells + gag peptide		gag peptide		no stimulation	
MVA control	0	2	0	4	1	2
MVA/48 (IP)	>500	>500	>500	>500	8	8
MVA/48 (ID)	12	5	49	33	4	2
MVA/48 (IM)	22	18	66	49	12	8

TABLE C

DNA prime and MVA/48 boost
Total ELISPOTS per Animal

Animal #	WEEKS						
	-2	2	6	10[2]	14[2]	20[2]	25[2]
RLw	4	731*	<	47	43	50	3905
RVl	5	997*	<	<	<	8	205
Roa	<[1]	<	1	<	<	<	245
RHc	<	<	<	<	<	<	535
Ryl	<	<	<	<	<	<	4130
RQk	<	46	<	<	<	<	630
RDr	<	<	<	14	<	<	1965
RZc	<	5	<	58	<	<	925
RSf	<	118	<	<	<	20	5570
Ras	<	69	<	<	<	<	1435
Total	9	1966	1	119	43	78	19545
Geo Mean	4.5	105.3	1.0	33.7	43.0	20.0	1147.7

DNA primes were at 0 and 8 weeks and MVA/48 boost was at 24 weeks

[1] < = Background (2x the number of ELISPOTs in the unstimulated control + 10)
[2]Costimulatory antibodies were added to the ELISPOT incubations
* Animals from this bleed date exhibited higher than usual ELISPOTs.

TABLE D

Antibody response following immunization of macaques with MVA/SHIV KB9

Animal #	Route	KB9 env	KB9 env elisa		SHIV-89.6	SHIV-89.6P	SHIV-89.6	SHIV-89.6P
		ELISA titer	average	std dev.	Nab titer	Nab titer	# pos animals	# pos animals
598	tonsil	25,600	31,086	20,383	<20	<20	3	2
601	"	51,200			<20	<20		
606	"	25,600			<20	<20		
642	"	51,200			75	31		
646	"	51,200			61	48		
653	"	6,400			<20	<20		
654	"	6,400			22	<20		
602	i.d.	25,600	18,800	15,341	38	<20	2	4
604	"	12,800			<20	262		
608	"	3,200			20	66		
637	"	12,800			<20	35		
638	"	51,200			<20	<20		
645	"	25,600			<20	<20		
647	"	12,800			32	162		
650	"	6,400			<20	<20		
599	i.m.	6,400	17,000	16,516	<20	<20	0	3
600	"	6,400			<20	29		
609	"	6,400			<20	<20		
639	"	51,200			<20	85		
640	"	12,800			<20	<20		
641	"	25,600			<20	41		
649	"	1,600			<20	<20		
651	"	25,600			20	<20		
603	Control	<100	<100		<20	<20	0	0
605	"	<100			<20	<20		
607	"	<100			<20	<20		
643	"	<100			<20	<20		
644	"	<100			<20	<20		
648	"	<100			<20	<20		
652	"	<100			<20	<20		

TABLE E

Frequencies of gag CM9-specific CD3/CD8 T cells following immunization of macaques with MVA/SHIV KB9

Animal #	Route	Virus	pre-bleed	1a	1b	1d	2a	2b	2c	2d
598	Tonsil	MVA/KB9	0.018	**0.41**	**0.79**	**0.25**	**2.64**	**1.13**	**0.51**	**0.21**
601	"	"	0.071	**0.34**	**0.38**	**0.27**	**0.83**	**0.7**	**0.36**	0.039
646	"	"	0.022	**0.68**	**0.76**	**0.43**	**1.12**	**0.91**	**0.53**	**0.15**
653	"	"	0.041	**0.69**	**0.85**	**0.53**	**0.68**	**0.49**	**0.47**	**0.3**
648	"	MVA		0.033	0.039		0.022	0.058	0.033	0.013
602	i.d.	MVA/KB9	0.019	**0.17**	**0.92**	**0.5**	**0.95**	**0.59**	**0.5**	**0.2**
604	"	"	0.013	**0.11**	**0.38**	**0.32**	**0.44**	**0.38**	**0.19**	**0.25**
650	"	"	0.095	**0.17**	**0.6**	**0.23**	**2.87**	**1.12**	**0.9**	**0.16**
647	"	"	0.032	**0.22**	**0.38**	**0.14**	**0.84**	**0.91**	**0.34**	**0.17**
652	"	MVA		0.041	0.038	0.059	0.025	0.022	0.026	0.055
599	i.m.	MVA/KB9		0.081	**0.31**	0.082		0.12	0.054	0.11
600	"	"	0.034	**0.15**	**0.41**	**0.17**	**0.29**	**0.27**	**0.16**	0.049
649	"	"	0.00486	**0.35**	**1.34**	**0.56**	**2.42**	**0.77**	**0.69**	0.22
651	"	"	0.049	**0.12**	**0.69**	**0.25**	**1.01**	**0.32**	**0.24**	0.22
603	"	MVA		0.024	0.087	0.073		0.082	0.027	0.17

TABLE F

Frequencies of specific T cells following immunization of macaques with MVA/SHIV KB9

	Gag specific			Tat specific			Nef specific			Env specific			Total
Study groups	# responding animals	Total # spots	average # spots	# responding animals	total # spots	average # spots	# responding animals	total # spots	average # spots	# responding animals	total spots	Average # spots	# responding animals
tonsil 1a	4/6	1325	221	0/6	0	0	3/6	195	33	6/6	8760	1460	6/6
tonsil 2a	5/6	1405	234	0/6	0	0	1/6	560	93	6/6	4485	748	6/6
i.d. 1a	7/7	1335	191	0/7	0	0	2/7	215	31	7/7	7320	1046	7/7
i.d. 2a	4/7	755	108	0/7	0	0	1/7	55	8	7/7	2700	386	7/7
i.m. 1a	7/7	925	132	1/7	60	9	3/7	180	26	7/7	5490	784	7/7
i.m. 2a	4/7	250	36	0/7	0	0	0/7	0	0	6/7	2205	315	6/7

*****

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention. All patents, patent applications and publications referred to above are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a recombinant MVA virus expressing an HIV *env*, *gag*, and *pol* gene or modified gene thereof for production of an HIV Env, Gag, and Pol antigen by expression from said recombinant MVA virus, wherein said HIV *env* gene is modified to encode an HIV Env protein composed of gp120 and the membrane-spanning and ectodomain of gp41 but lacking part or all of the cytoplasmic domain of gp41, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein said HIV *pol* gene or modified gene thereof is modified to inactivate reverse transcriptase and integrase.

3. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade A.

4. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade B.

5. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade C.

6. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade D.

7. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade E.

8. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade F.

9. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade G.

10. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade H.

11. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade J.

12. The pharmaceutical composition of claim 1 wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is inserted at the site of deletion III within the MVA genome.

13. The pharmaceutical composition of claim 1 wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is under transcriptional initiation regulation of a H5-like early/late vaccinia virus promoter.

14. The pharmaceutical composition of claim 1 wherein recombinant MVA virus additionally expresses an additional HIV gene or modified gene thereof for production of an HIV antigen by expression from said recombinant MVA virus, wherein said additional HIV gene is a member selected from the group consisting of *vif, vpr, tat, rev, vpu*, and *nef*.

15. MVA/HIV48 comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

16. pLW-48 having SEQ ID NO:1.

17. A plasmid transfer vector having the sequence of pLW-48 (SEQ ID NO:1) excluding the HIV *env*, *gag*, and *pol* genes.

18. pLW-48 (SEQ ID NO:1) wherein the HIV *env*, *gag*, and *pol* genes have a sequence taken from another clade.

19. A poxvirus comprising a promoter selected from the group consisting of m7.5 promoter having SEQ ID NO:10, Psyn II promoter having SEQ ID NO:2, Psyn III promoter having SEQ ID NO:11, Psyn IV promoter having SEQ ID NO:12, and Psyn V promoter having SEQ ID NO:13.

20. A method of boosting a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a composition of any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen previously primed in the primate is boosted.

21. A method of inducing a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a composition of any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen in the primate is induced.

22. A method of inducing a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a priming composition comprising nucleic acid encoding said antigen and then provision in the primate of a boosting composition which comprises any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen is induced.

23. The method of any of Claims 20-22, wherein the primate is a human.

24. The method of any of Claims 20-22, wherein administration of the recombinant MVA virus is by needleless injection.

25. The method of Claim 22, wherein the priming composition comprises plasmid DNA encoding said antigen.

26. A method of making a composition of any of claims 1-15 comprising preparing a plasmid transfer vector encoding an HIV *env*, *gag*, and *pol* gene or modified gene thereof, wherein said HIV *env* gene is modified to encode an HIV Env protein composed of gp120 and the membrane-spanning and ectodomain of gp41 but lacking part or all of the cytoplasmic domain of gp41, and recombining said plasmid transfer vector with a MVA virus to produce a composition of any of claims 1-15.

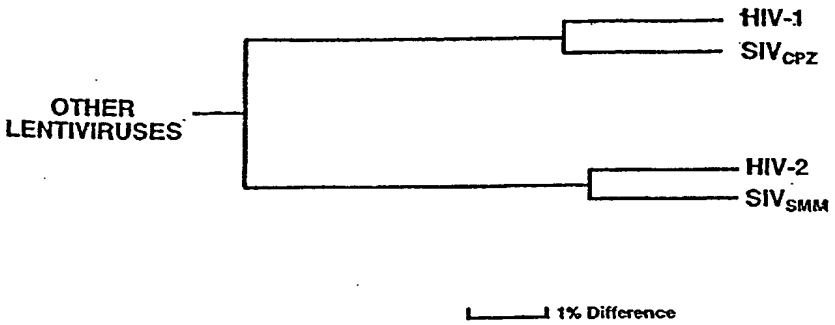


Figure I

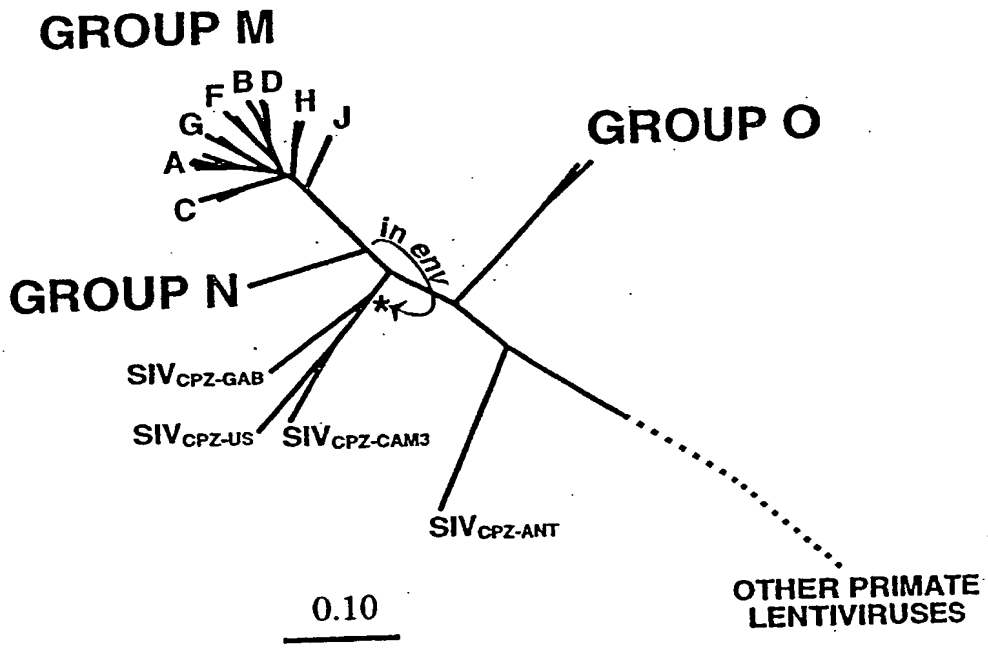
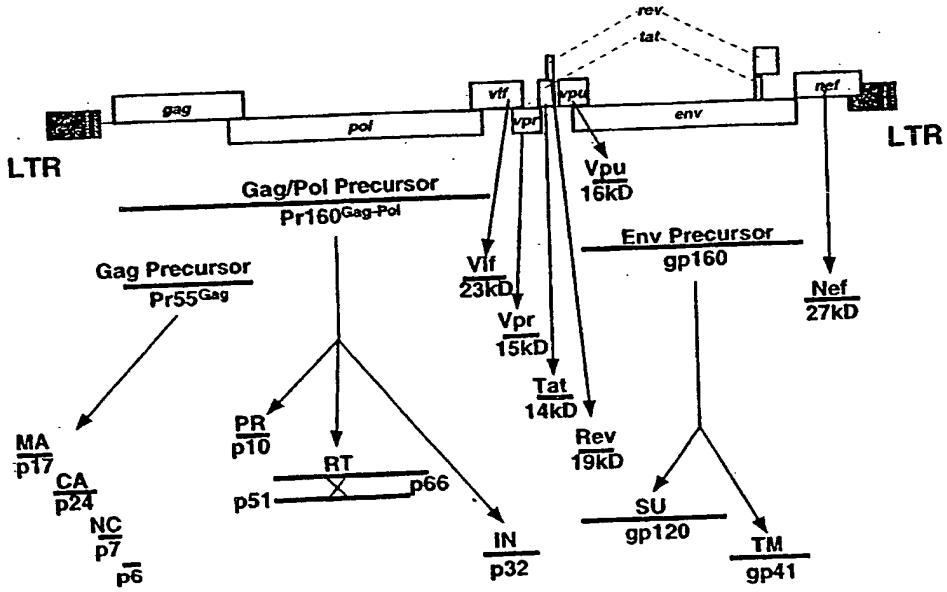

GROUP M
F
B
D
H
J
G
A
C
GROUP O
in env
GROUP N
SIVCPZ-GAB
SIVCPZ-US
SIVCPZ-CAM3
SIVCPZ-ANT
OTHER PRIMATE LENTIVIRUSES
0.10

Figure II

Chemokine coreceptor used	PBMC replication	Macrophage replication	T-cell-line replication	Replicative phenotype	Syncytium-inducing phenotype
X4	+	-	+	Rapid/high	++
R5	+	+	-	Slow/low	-
R5/X4	+	+	+	Rapid/high	+

Figure III


rev
tat
gag
pol
vif
vpr
vpu
env
nef
LTR
LTR
Gag/Pol Precursor
Pr160Gag-Pol
Gag Precursor
Pr55Gag
Vpu
16kD
Env Precursor
gp160
Vif
23kD
Vpr
15kD
Tat
14kD
Rev
19kD
Nef
27kD
MA
p17
CA
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p10
RT
p51
p66
IN
p32
SU
gp120
TM
gp41

Figure IV

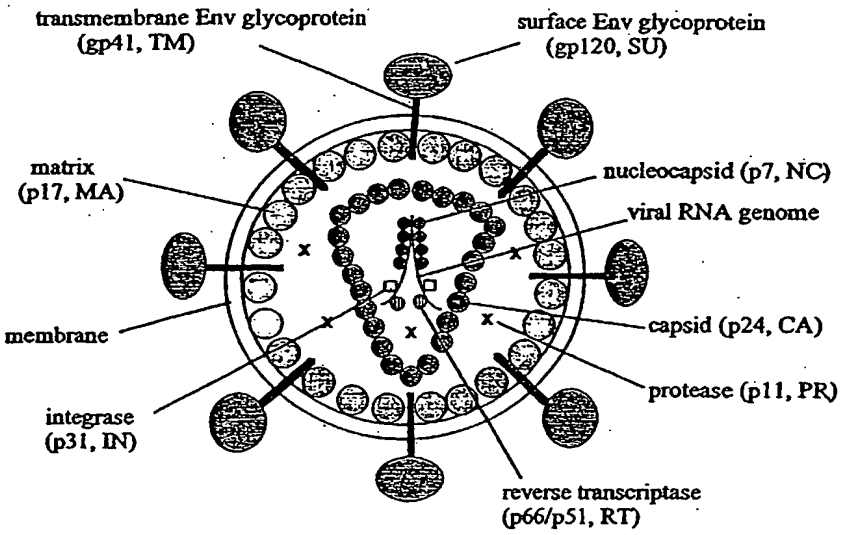
transmembrane Env glycoprotein
(gp41, TM)
surface Env glycoprotein
(gp120, SU)
matrix
(p17, MA)
nucleocapsid (p7, NC)
viral RNA genome
membrane
capsid (p24, CA)
protease (p11, PR)
integrase
(p31, IN)
reverse transcriptase
(p66/p51, RT)

Figure V

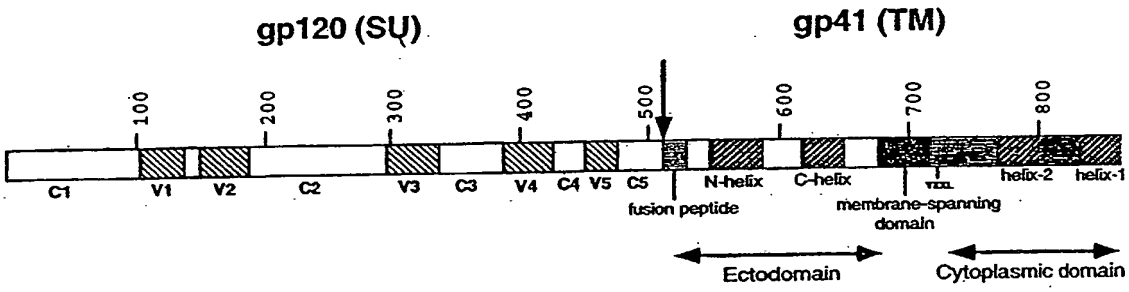
gp120 (SU)
gp41 (TM)
100
200
300
400
500
600
700
800
C1
V1
V2
C2
V3
C3
V4
C4
V5
C5
fusion peptide
N-helix
C-helix
membrane-spanning domain
helix-2
helix-1
Ectodomain
Cytoplasmic domain

Figure VI

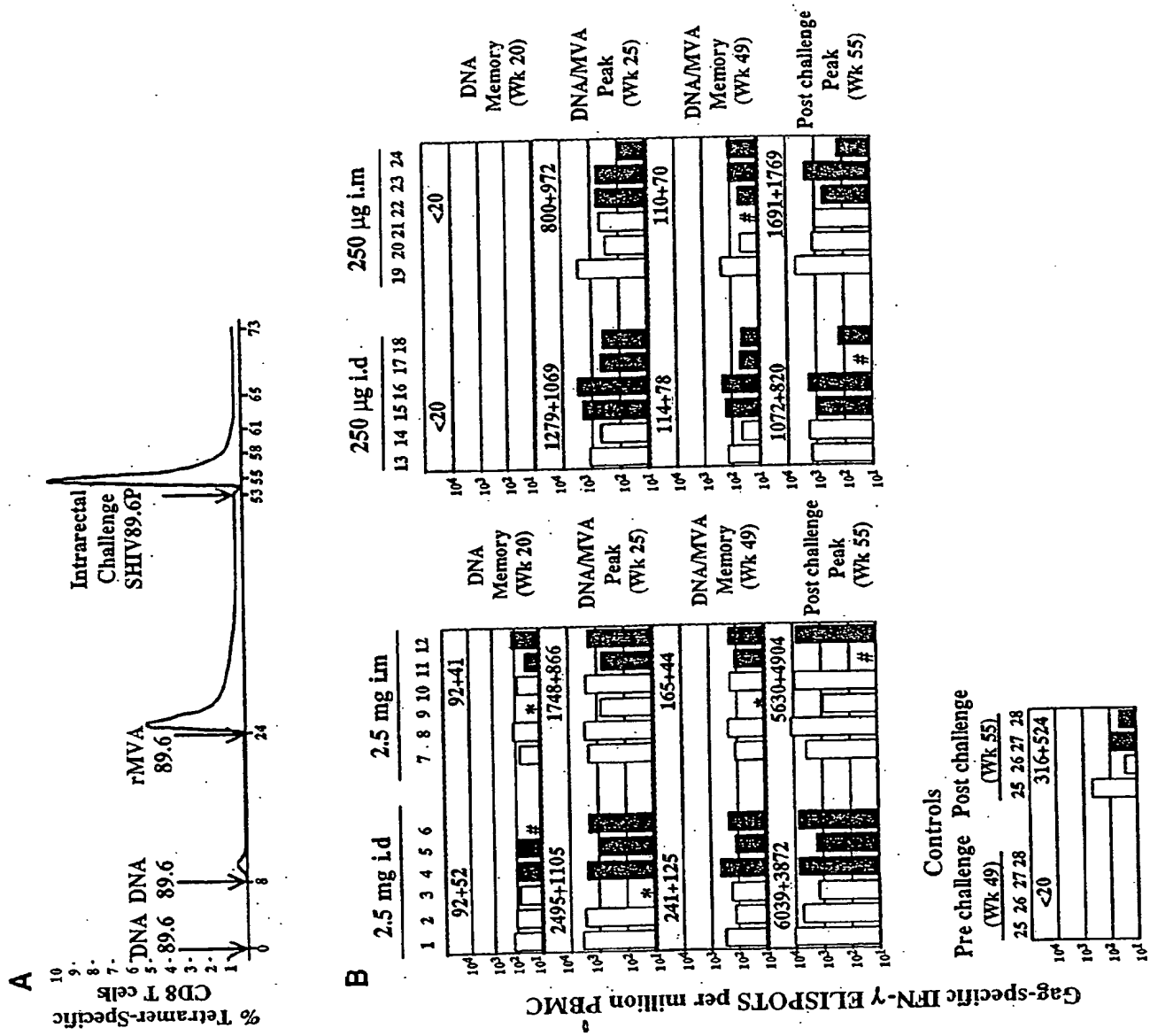
A
% Tetramer-Specific CD8 T cells
DNA 89.6
DNA 89.6
rMVA 89.6
Intrarectal Challenge SHIV89.6P
B
2.5 mg i.d
2.5 mg im
250 µg i.d
250 µg i.m
DNA Memory (Wk 20)
DNA/MVA Peak (Wk 25)
DNA/MVA Memory (Wk 49)
Post challenge Peak (Wk 55)
92+52
92+41
2495+1105
1748+866
241+125
165+44
6039+3872
5630+4904
<20
<20
1279+1069
800+972
114+78
110+70
1072+820
1691+1769
Controls
Pre challenge (Wk 49)
Post challenge (Wk 55)
316+524
Gag-specific IFN-γ ELISPOTS per million PBMC

Figure 1

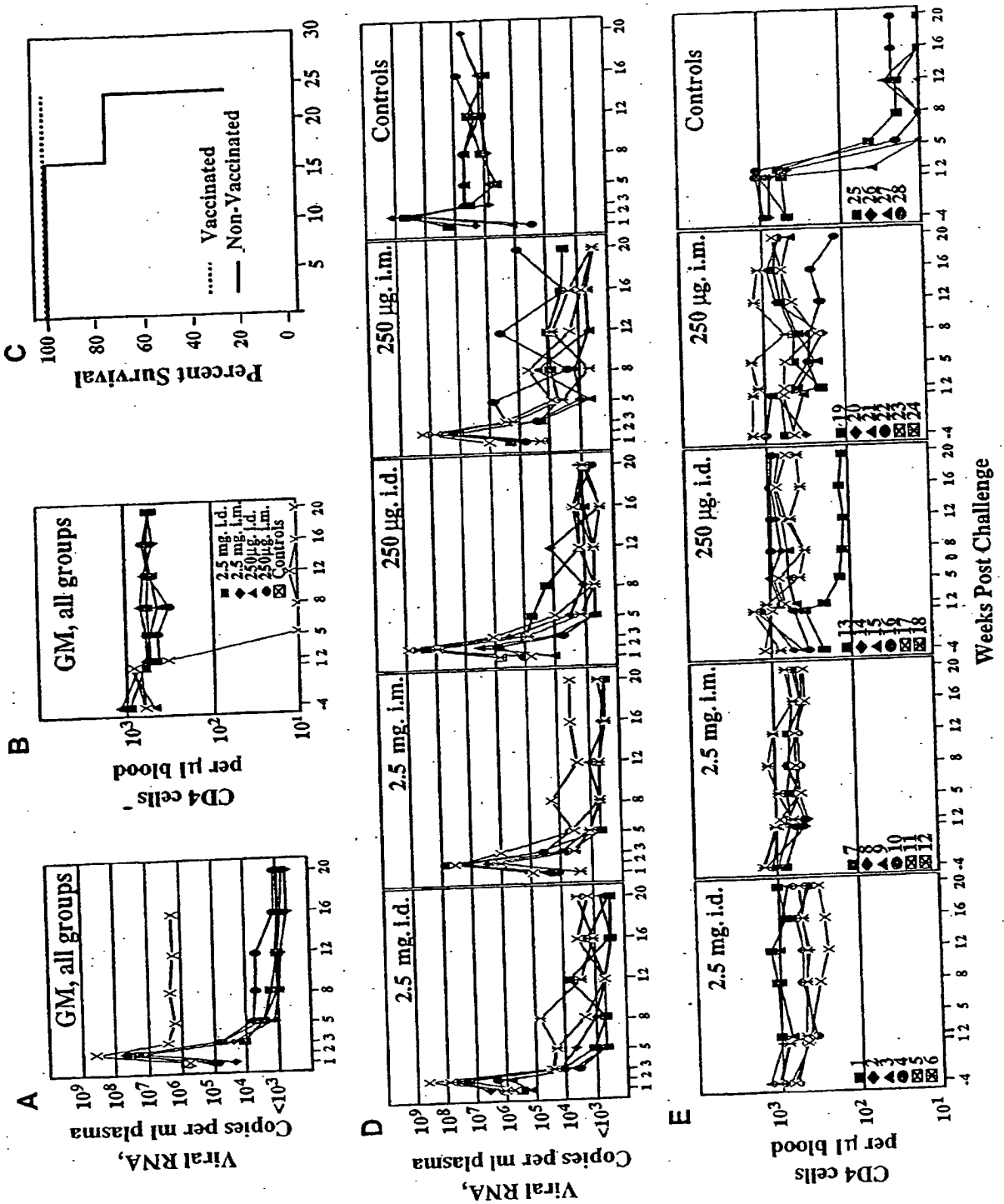
A
GM, all groups
Viral RNA, Copies per ml plasma
B
GM, all groups
CD4 cells per µl blood
2.5 mg. i.d.
2.5 mg. i.m.
250µg. i.d.
250µg. i.m.
Controls
C
Percent Survival
Vaccinated
Non-Vaccinated
D
Controls
250 µg. i.m.
250 µg. i.d.
2.5 mg. i.m.
2.5 mg. i.d.
Viral RNA, Copies per ml plasma
E
Controls
250 µg. i.m.
250 µg. i.d.
2.5 mg. i.m.
2.5 mg. i.d.
CD4 cells per µl blood
Weeks Post Challenge

Figure 2

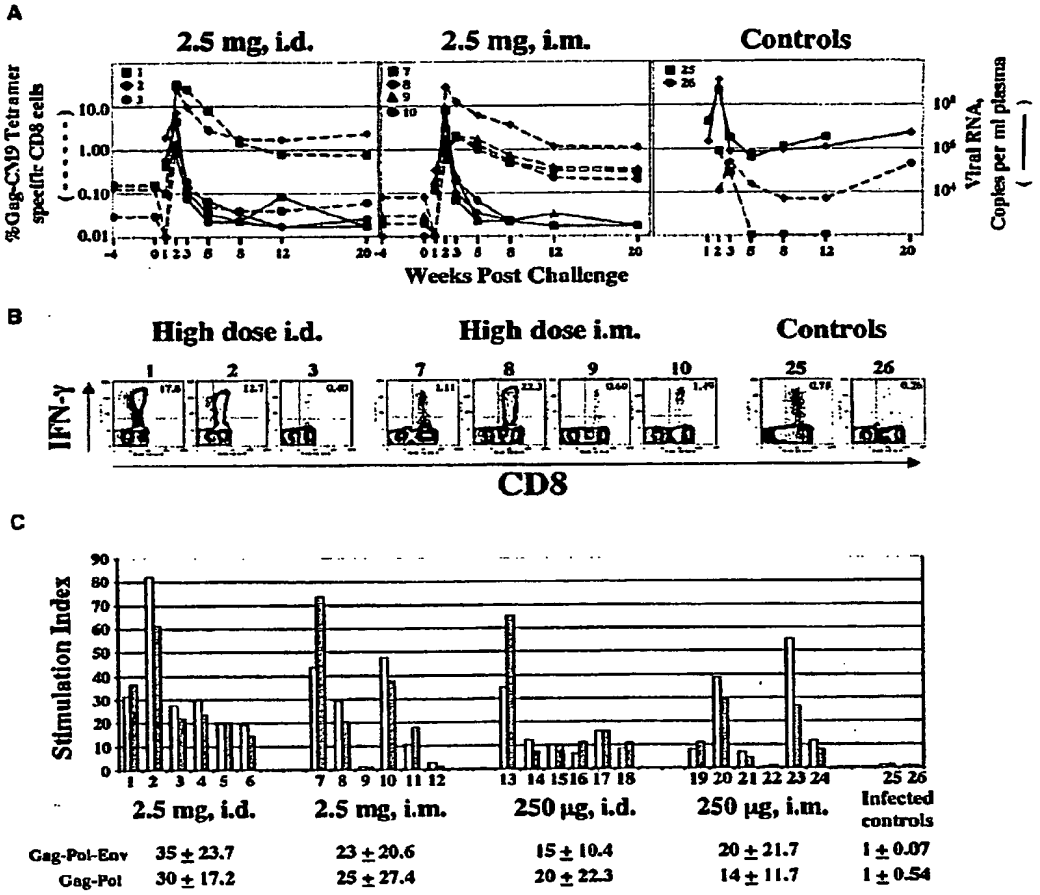
A
2.5 mg, i.d.
2.5 mg, i.m.
Controls
%Gag-CM9 Tetramer specific CD8 cells
Viral RNA, Copies per ml plasma
Weeks Post Challenge
B
High dose i.d.
High dose i.m.
Controls
IFN-γ
CD8
C
Stimulation Index
2.5 mg, i.d.
2.5 mg, i.m.
250 µg, i.d.
250 µg, i.m.
Infected controls
Gag-Pol-Env 35 ± 23.7 23 ± 20.6 15 ± 10.4 20 ± 21.7 1 ± 0.07
Gag-Pol 30 ± 17.2 25 ± 27.4 20 ± 22.3 14 ± 11.7 1 ± 0.54

Figure 3

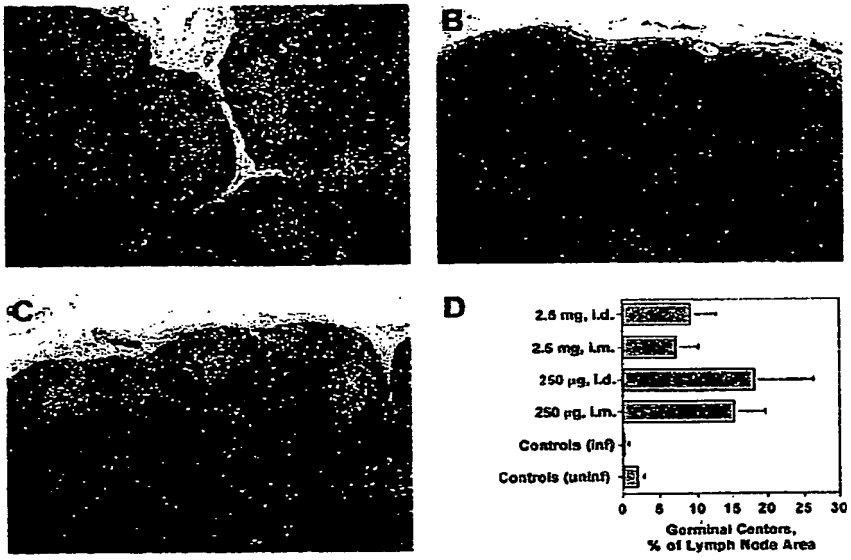
B
C
D
2.5 mg, i.d.
2.5 mg, i.m.
250 µg, i.d.
250 µg, i.m.
Controls (inf)
Controls (uninf)
0
5
10
15
20
25
30
Germinal Centers,
% of Lymph Node Area

Figure 4

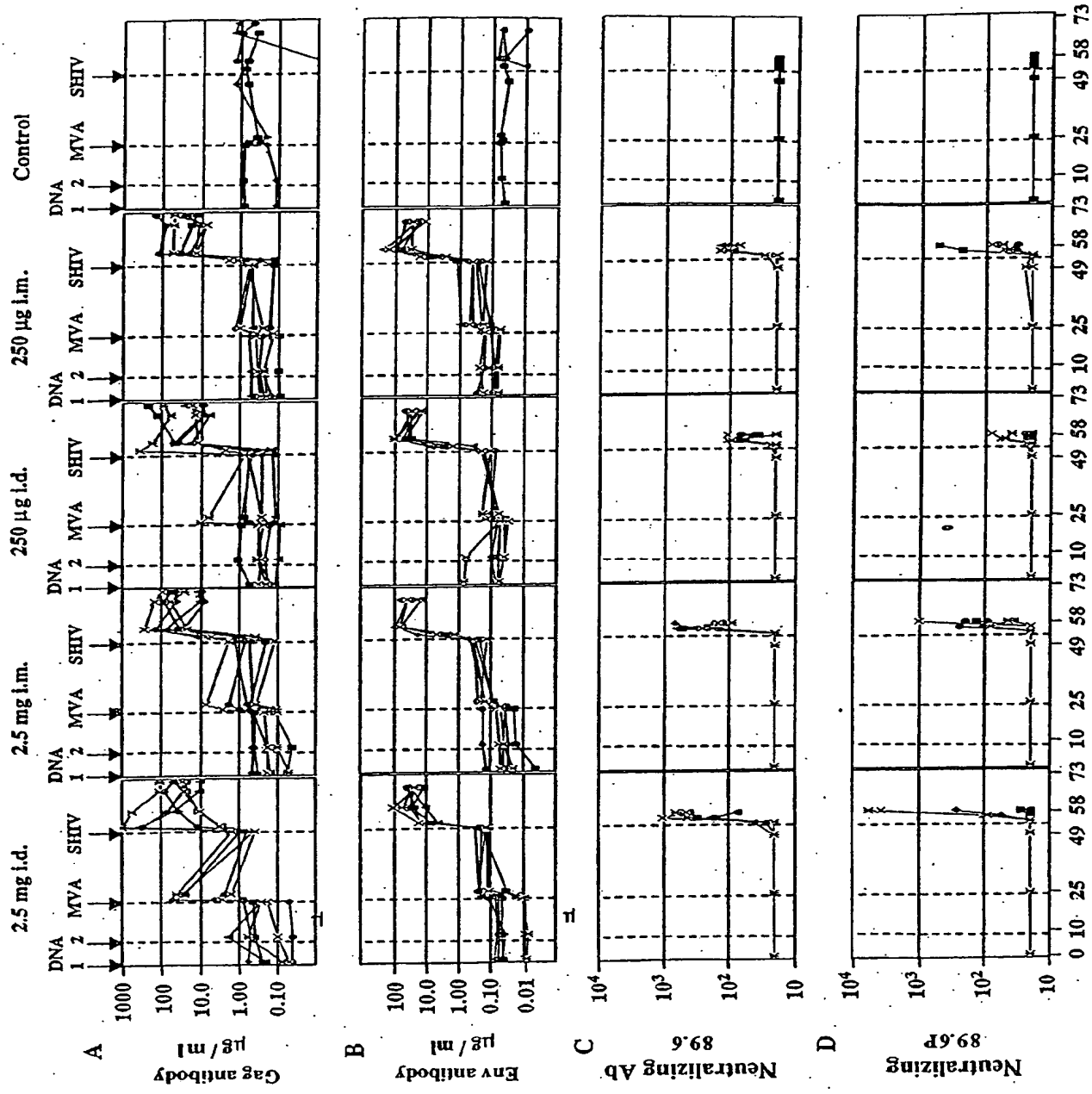
2.5 mg i.d.
2.5 mg i.m.
250 µg i.d.
250 µg i.m.
Control
DNA
MVA
SHIV
A
Gag antibody µg / ml
B
Env antibody µg / ml
C
Neutralizing Ab 89.6
D
Neutralizing 89.6P
Time in weeks

Figure 5

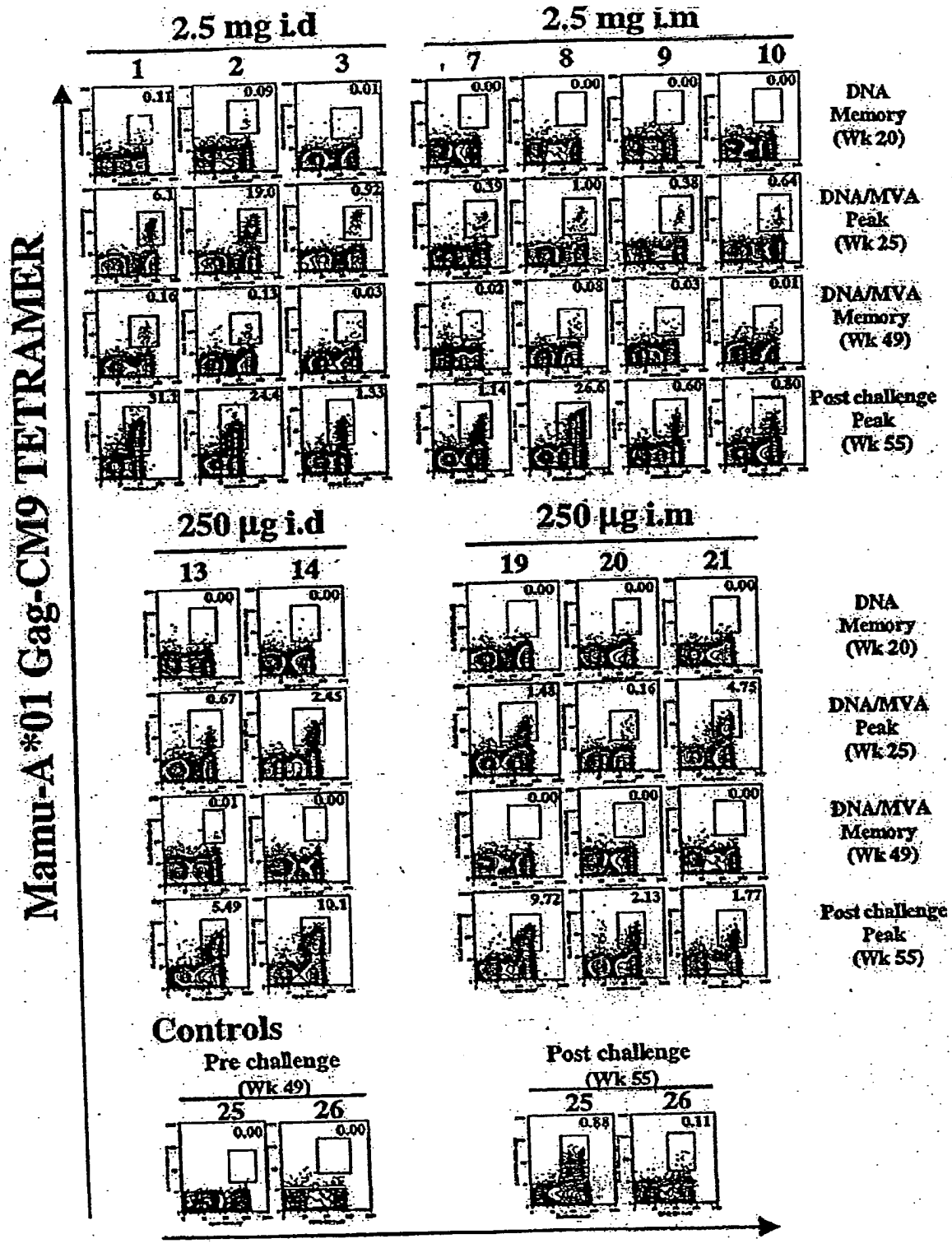
2.5 mg i.d
2.5 mg i.m
1
2
3
7
8
9
10
DNA Memory (Wk 20)
DNA/MVA Peak (Wk 25)
DNA/MVA Memory (Wk 49)
Post challenge Peak (Wk 55)
250 µg i.d
250 µg i.m
13
14
19
20
21
Mamu-A*01 Gag-CM9 TETRAMER
Controls
Pre challenge (Wk 49)
Post challenge (Wk 55)
25
26

Figure 6

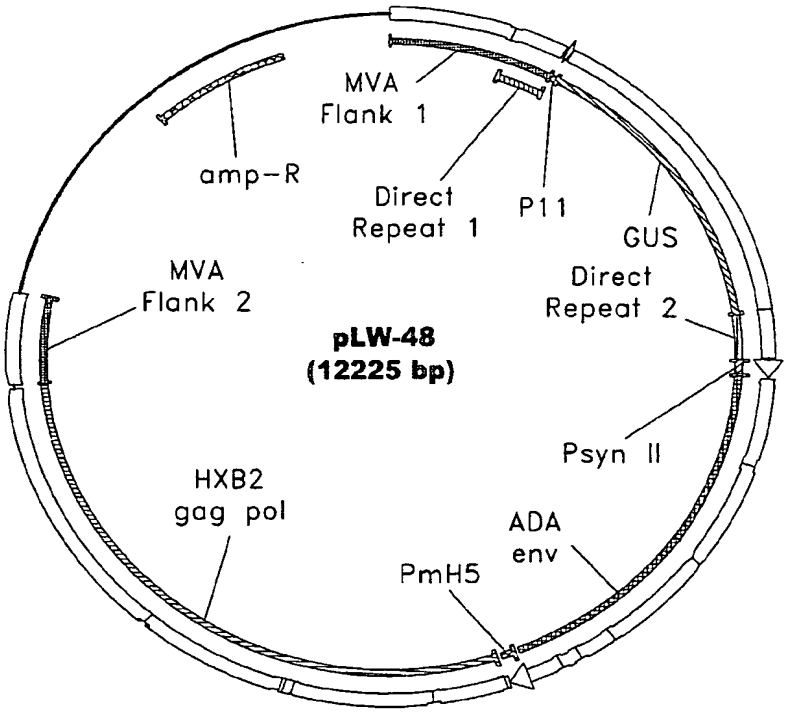
MVA
Flank 1
amp-R
Direct
Repeat 1
P11
GUS
MVA
Flank 2
Direct
Repeat 2
pLW-48
(12225 bp)
Psyn II
HXB2
gag pol
ADA
env
PmH5

FIG. A,

```
1   GAATTCGTTG GTGGTCGCCA TGGATGGTGT TATTGTATAC TGTCTAAACG CGTTAGTAAA ACATGGCGAG
    CTTAAGCAAC CACCAGCGGT ACCTACCACA ATAACATATG ACAGATTTGC GCAATCATTT TGTACCGCTC

71  GAAATAAATC ATATAAAAAA TGATTTCATG ATTAAACCAT GTTGTGAAAA AGTCAAGAAC GTTCACATTG
    CTTTATTTAG TATATTTTTT ACTAAAGTAC TAATTTGGTA CAACACTTTT TCAGTTCTTG CAAGTGTAAC

141 GCGGACAATC TAAAAACAAT ACAGTGATTG CAGATTTGCC ATATATGGAT AATGCGGTAT CCGATGTATG
    CGCCTGTTAG ATTTTTGTTA TGTCACTAAC GTCTAAACGG TATATACCTA TTACGCCATA GGCTACATAC

211 CAATTCACTG TATAAAAAGA ATGTATCAAG AATATCCAGA TTTGCTAATT TGATAAAGAT AGATGACGAT
    GTTAAGTGAC ATATTTTTCT TACATAGTTC TTATAGGTCT AAACGATTAA ACTATTTCTA TCTACTGCTA

281 GACAAGACTC CTACTGGTGT ATATAATTAT TTTAAACCTA AAGATGCCAT TCCTGTTATT ATATCCATAG
    CTGTTCTGAG GATGACCACA TATATTAATA AAATTTGGAT TTCTACGGTA AGGACAATAA TATAGGTATC

351 GAAAGGATAG AGATGTTTGT GAACTATTAA TCTCATCTGA TAAAGCGTGT GCGTGTATAG AGTTAAATTC
    CTTTCCTATC TCTACAAACA CTTGATAATT AGAGTAGACT ATTTCGCACA CGCACATATC TCAATTTAAG
```

FIG. A_2

421 ATATAAAGTA GCCATTCTTC CCATGGATGT TTCCTTTTTT ACCAAAGGAA ATGCATCATT GATTATTCTC
TATATTTCAT CGGTAAGAAG GGTACCTACA AAGGAAAAAA TGGTTTCCTT TACGTAGTAA CTAATAAGAG

491 CTGTTTGATT TCTCTATCGA TGCGGCACCT CTCTTAAGAA GTGTAACCGA TAATAATGTT ATTATATCTA
GACAAACTAA AGAGATAGCT ACGCCGTGGA GAGAATTCTT CACATTGGCT ATTATTACAA TAATATAGAT

561 GACACCAGCG TCTACATGAC GAGCTTCCGA GTTCCAATTG GTTCAAGTTT TACATAAGTA TAAAGTCCGA
CTGTGGTCGC AGATGTACTG CTCGAAGGCT CAAGGTTAAC CAAGTTCAAA ATGTATTCAT ATTTCAGGCT

631 CTATTGTTCT ATATTATATA TGGTTGTTGA TGGATCTGTG ATGCATGCAA TAGCTGATAA TAGAACTTAC
GATAACAAGA TATAATATAT ACCAACAACT ACCTAGACAC TACGTACGTT ATCGACTATT ATCTTGAATG

701 GCAAATATTA GCAAAAATAT ATTAGACAAT ACTACAATTA ACGATGAGTG TAGATGCTGT TATTTTGAAC
CGTTTATAAT CGTTTTTATA TAATCTGTTA TGATGTTAAT TGCTACTCAC ATCTACGACA ATAAAACTTG

FIG. $A_{2\ cont.}$

771 CACAGATTAG GATTCTTGAT AGAGATGAGA TGCTCAATGG ATCATCGTGT GATATGAACA GACATTGTAT
GTGTCTAATC CTAAGAACTA TCTCTACTCT ACGAGTTACC TAGTAGCACA CTATACTTGT CTGTAACATA

841 TATGATGAAT TTACCTGATG TAGGCGAATT TGGATCTAGT ATGTTGGGGA AATATGAACC TGACATGATT
ATACTACTTA AATGGACTAC ATCCGCTTAA ACCTAGATCA TACAACCCCT TTATACTTGG ACTGTACTAA

911 AAGATTGCTC TTTCGGTGGC TGGGTACCAG GCGCGCCTTT CATTTTGTTT TTTTCTATGC TATAAATGGT
TTCTAACGAG AAAGCCACCG ACCCATGGTC CGCGCGGAAA GTAAAACAAA AAAAGATACG ATATTTACCA

981 ACGTCCTGTA GAAACCCCAA CCCGTGAAAT CAAAAAACTC GACGGCCTGT GGGCATTCAG TCTGGATCGC
TGCAGGACAT CTTTGGGGTT GGGCACTTTA GTTTTTTGAG CTGCCGGACA CCCGTAAGTC AGACCTAGCG

1051 GAAAACTCTG GAATTGATCA GCGTTGGTGG GAAAGCGCGT TACAAGAAAG CCGGGCAATT GCTGTGCCAG
CTTTTGACAC CTTAACTAGT CGCAACCACC CTTTCGCGCA ATGTTCTTTC GGCCCGTTAA CGACACGGTC

FIG. $A_{2\ cont.}$

```
1121  GCAGTTTTAA CGATCAGTTC GCCGATGCAG ATATTCGTAA TTATGCGGGC AACGTCTGGT ATCAGCGCGA
      CGTCAAAATT GCTAGTCAAG CGGCTACGTC TATAAGCATT AATACGCCCG TTGCAGACCA TAGTCGCGCT

1191  AGTCTTTATA CCGAAAGGTT GGGCAGGCCA GCGTATCGTG CTGCGTTTCG ATGCGGTCAC TCATTACGGC
      TCAGAAATAT GGCTTTCCAA CCCGTCCGGT CGCATAGCAC GACGCAAAGC TACGCCAGTG AGTAATGCCG

1261  AAAGTGTGGG TCAATAATCA GGAAGTGATG GAGCATCAGG GCGGCTATAC GCCATTTGAA GCCGATGTCA
      TTTCACACCC AGTTATTAGT CCTTCACTAC CTCGTAGTCC CGCCGATATG CGGTAAACTT CGGCTACAGT

1331  CGCCGTATGT TATTGCCGGG AAAAGTGTAC GTATCACCGT TTGTGTGAAC AACGAACTGA ACTGGCAGAC
      GCGGCATACA ATAACGGCCC TTTTCACATG CATAGTGGCA AACACACTTG TTGCTTGACT TGACCGTCTG

1401  TATCCCGCCG GGAATGGTGA TTACCGACGA AAACGGCAAG AAAAAGCAGT CTTACTTCCA TGATTTCTTT
      ATAGGGCGGC CCTTACCACT AATGGCTGCT TTTGCCGTTC TTTTTCGTCA GAATGAAGGT ACTAAAGAAA

1471  AACTATGCCG GAATCCATCG CAGCGTAATG CTCTACACCA CGCCGAACAC CTGGGTGGAC GATATCACCG
      TTGATACGGC CTTAGGTAGC GTCGCATTAC GAGATGTGGT GCGGCTTGTG GACCCACCTG CTATAGTGGC
```

FIG. $A_{2\ cont.}$

1541 TGGTGACGCA TGTCGCGCAA GACTGTAACC ACGCGTCTGT TGACTGGCAG GTGGTGGCCA ATGGTGATGT
ACCACTGCGT ACAGCGCGTT CTGACATTGG TGCGCAGACA ACTGACCGTC CACCACCGGT TACCACTACA

1611 CAGCGTTGAA CTGCGTGATG CGGATCAACA GGTGGTTGCA ACTGGACAAG GCACTAGCGG GACTTTGCAA
GTCGCAACTT GACGCACTAC GCCTAGTTGT CCACCAACGT TGACCTGTTC CGTGATCGCC CTGAAACGTT

1681 GTGGTGAATC CGCACCTCTG GCAACCGGGT GAAGGTTATC TCTATGAACT GTGCGTCACA GCCAAAAGCC
CACCACTTAG GCGTGGAGAC CGTTGGCCCA CTTCCAATAG AGATACTTGA CACGCAGTGT CGGTTTTCGG

1751 AGACAGAGTG TGATATCTAC CCGCTTCGCG TCGGCATCCG GTCAGTGGCA GTGAAGGGCG AACAGTTCCT
TCTGTCTCAC ACTATAGATG GGCGAAGCGC AGCCGTAGGC CAGTCACCGT CACTTCCCGC TTGTCAAGGA

1821 GATTAACCAC AAACCGTTCT ACTTTACTGG CTTTGGTCGT CATGAAGATG CGGACTTGCG TGGCAAAGGA
CTAATTGGTG TTTGGCAAGA TGAAATGACC GAAACCAGCA GTACTTCTAC GCCTGAACGC ACCGTTTCCT

1891 TTCGATAACG TGCTGATGGT GCACGACCAC GCATTAATGG ACTGGATTGG GGCCAACTCC TACCGTACCT
AAGCTATTGC ACGACTACCA CGTGCTGGTG CGTAATTACC TGACCTAACC CCGGTTGAGG ATGGCATGGA

FIG. $A_{2\ cont.}$

1961 CGCATTACCC TTACGCTGAA GAGATGCTCG ACTGGGCAGA TGAACATGGC ATCGTGGTGA TTGATGAAAC
GCGTAATGGG AATGCGACTT CTCTACGAGC TGACCCGTCT ACTTGTACCG TAGCACCACT AACTACTTTG

2031 TGCTGCTGTC GGCTTTAACC TCTCTTTAGG CATTGGTTTC GAAGCGGGCA ACAAGCCGAA AGAACTGTAC
ACGACGACAG CCGAAATTGG AGAGAAATCC GTAACCAAAG CTTCGCCCGT TGTTCGGCTT TCTTGACATG

2101 AGCGAAGAGG CAGTCAACGG GGAAACTCAG CAAGCGCACT TACAGGCGAT TAAAGAGCTG ATAGCGCGTG
TCGCTTCTCC GTCAGTTGCC CCTTTGAGTC GTTCGCGTGA ATGTCCGCTA ATTTCTCGAC TATCGCGCAC

2171 ACAAAAACCA CCCAAGCGTG GTGATGTGGA GTATTGCCAA CGAACCGGAT ACCCGTCCGC AAGGTGCACG
TGTTTTTGGT GGGTTCGCAC CACTACACCT CATAACGGTT GCTTGGCCTA TGGGCAGGCG TTCCACGTGC

2241 GGAATATTTC GCGCCACTGG CGGAAGCAAC GCGTAAACTC GACCCGACGC GTCCGATCAC CTGCGTCAAT
CCTTATAAAG CGCGGTGACC GCCTTCGTTG CGCATTTGAG CTGGGCTGCG CAGGCTAGTG GACGCAGTTA

2311 GTAATGTTCT GCGACGCTCA CACCGATACC ATCAGCGATC TCTTTGATGT GCTGTGCCTG AACCGTTATT
CATTACAAGA CGCTGCGAGT GTGGCTATGG TAGTCGCTAG AGAAACTACA CGACACGGAC TTGGCAATAA

FIG. $A_{2\ cont.}$

2381 ACGGATGGTA TGTCCAAAGC GGCGATTTGG AAACGGCAGA GAAGGTACTG GAAAAAGAAC TTCTGGCCTG
TGCCTACCAT ACAGGTTTCG CCGCTAAACC TTTGCCGTCT CTTCCATGAC CTTTTTCTTG AAGACCGGAC

2451 GCAGGAGAAA CTGCATCAGC CGATTATCAT CACCGAATAC GGCGTGGATA CGTTAGCCGG GCTGCACTCA
CGTCCTCTTT GACGTAGTCG GCTAATAGTA GTGGCTTATG CCGCACCTAT GCAATCGGCC CGACGTGAGT

2521 ATGTACACCG ACATGTGGAG TGAAGAGTAT CAGTGTGCAT GGCTGGATAT GTATCACCGC GTCTTTGATC
TACATGTGGC TGTACACCTC ACTTCTCATA GTCACACGTA CCGACCTATA CATAGTGGCG CAGAAACTAG

2591 GCGTCAGCGC CGTCGTCGGT GAACAGGTAT GGAATTTCGC CGATTTTGCG ACCTCGCAAG GCATATTGCG
CGCAGTCGCG GCAGCAGCCA CTTGTCCATA CCTTAAAGCG GCTAAAACGC TGGAGCGTTC CGTATAACGC

2661 CGTTGGCGGT AACAAGAAAG GGATCTTCAC TCGCGACCGC AAACCGAAGT CGGCGGCTTT TCTGCTGCAA
GCAACCGCCA TTGTTCTTTC CCTAGAAGTG AGCGCTGGCG TTTGGCTTCA GCCGCCGAAA AGACGACGTT

2731 AAACGCTGGA CTGGCATGAA CTTCGGTGAA AAACCGCAGC AGGGAGGCAA ACAATGAGAG CTCGGTTGTT
TTTGCGACCT GACCGTACTT GAAGCCACTT TTTGGCGTCG TCCCTCCGTT TGTTACTCTC GAGCCAACAA

FIG. $A_{2\ cont.}$

2801 GATGGATCTG TGATGCATGC AATAGCTGAT AATAGAACTT ACGCAAATAT TAGCAAAAAT ATATTAGACA
CTACCTAGAC ACTACGTACG TTATCGACTA TTATCTTGAA TGCGTTTATA ATCGTTTTTA TATAATCTGT

2871 ATACTACAAT TAACGATGAG TGTAGATGCT GTTATTTTGA ACCACAGATT AGGATTCTTG ATAGAGATGA
TATGATGTTA ATTGCTACTC ACATCTACGA CAATAAAACT TGGTGTCTAA TCCTAAGAAC TATCTCTACT

2941 GATGCTCAAT GGATCATCGT GTGATATGAA CAGACATTGT ATTATGATGA ATTTACCTGA TGTAGGCGAA
CTACGAGTTA CCTAGTAGCA CACTATACTT GTCTGTAACA TAATACTACT TAAATGGACT ACATCCGCTT

3011 TTTGGATCTA GTATGTTGGG GAAATATGAA CCTGACATGA TTAAGATTGC TCTTTCGGTG GCTGGCGGCC
AAACCTAGAT CATACAACCC CTTTATACTT GGACTGTACT AATTCTAACG AGAAAGCCAC CGACCGCCGG

3081 CGCTCGAGTA AAAAATGAAA AAATATTCTA ATTTATAGGA CGGTTTTGAT TTTCTTTTTT TCTATGCTAT
GCGAGCTCAT TTTTTACTTT TTTATAAGAT TAAATATCCT GCCAAAACTA AAAGAAAAAA AGATACGATA

3151 AAATAATAAA TAGCGGCCGC ACCATGAAAG TGAAGGGGAT CAGGAAGAAT TATCAGCACT TGTGGAAATG
TTTATTATTT ATCGCCGGCG TGGTACTTTC ACTTCCCCTA GTCCTTCTTA ATAGTCGTGA ACACCTTTAC

FIG. $A_{2\ cont.}$

3221 GGGCATCATG CTCCTTGGGA TGTTGATGAT CTGTAGTGCT GTAGAAAATT TGTGGGTCAC AGTTTATTAT
CCCGTAGTAC GAGGAACCCT ACAACTACTA GACATCACGA CATCTTTTAA ACACCCAGTG TCAAATAATA

3291 GGGGTACCTG TGTGGAAAGA AGCAACCACC ACTCTATTTT GTGCATCAGA TGCTAAAGCA TATGATACAG
CCCCATGGAC ACACCTTTCT TCGTTGGTGG TGAGATAAAA CACGTAGTCT ACGATTTCGT ATACTATGTC

3361 AGGTACATAA TGTTTGGGCC ACACATGCCT GTGTACCCAC AGACCCCAAC CCACAAGAAG TAGTATTGGA
TCCATGTATT ACAAACCCGG TGTGTACGGA CACATGGGTG TCTGGGGTTG GGTGTTCTTC ATCATAACCT

3431 AAATGTGACA GAAAATTTTA ACATGTGGAA AAATAACATG GTAGAACAGA TGCATGAGGA TATAATCAGT
TTTACACTGT CTTTTAAAAT TGTACACCTT TTTATTGTAC CATCTTGTCT ACGTACTCCT ATATTAGTCA

3501 TTATGGGATC AAAGCCTAAA GCCATGTGTA AAATTAACCC CACTCTGTGT TACTTTAAAT TGCACTGATT
AATACCCTAG TTTCGGATTT CGGTACACAT TTTAATTGGG GTGAGACACA ATGAAATTTA ACGTGACTAA

3571 TGAGGAATGT TACTAATATC AATAATAGTA GTGAGGGAAT GAGAGGAGAA ATAAAAAACT GCTCTTTCAA
ACTCCTTACA ATGATTATAG TTATTATCAT CACTCCCTTA CTCTCCTCTT TATTTTTTGA CGAGAAAGTT

FIG. $A_{2\ cont.}$

```
3641  TATCACCACA AGCATAAGAG ATAAGGTGAA GAAAGACTAT GCACTTTTCT ATAGACTTGA TGTAGTACCA
      ATAGTGGTGT TCGTATTCTC TATTCCACTT CTTTCTGATA CGTGAAAAGA TATCTGAACT ACATCATGGT

3711  ATAGATAATG ATAATACTAG CTATAGGTTG ATAAATTGTA ATACCTCAAC CATTACACAG GCCTGTCCAA
      TATCTATTAC TATTATGATC GATATCCAAC TATTTAACAT TATGGAGTTG GTAATGTGTC CGGACAGGTT

3781  AGGTATCCTT TGAGCCAATT CCCATACATT ATTGTACCCC GGCTGGTTTT GCGATTCTAA AGTGTAAAGA
      TCCATAGGAA ACTCGGTTAA GGGTATGTAA TAACATGGGG CCGACCAAAA CGCTAAGATT TCACATTTCT

3851  CAAGAAGTTC AATGGAACAG GGCCATGTAA AAATGTCAGC ACAGTACAAT GTACACATGG AATTAGGCCA
      GTTCTTCAAG TTACCTTGTC CCGGTACATT TTTACAGTCG TGTCATGTTA CATGTGTACC TTAATCCGGT

3921  GTAGTGTCAA CTCAACTGCT GTTAAATGGC AGTCTAGCAG AAGAAGAGGT AGTAATTAGA TCTAGTAATT
      CATCACAGTT GAGTTGACGA CAATTTACCG TCAGATCGTC TTCTTCTCCA TCATTAATCT AGATCATTAA

3991  TCACAGACAA TGCAAAAAAC ATAATAGTAC AGTTGAAAGA ATCTGTAGAA ATTAATTGTA CAAGACCCAA
      AGTGTCTGTT ACGTTTTTTG TATTATCATG TCAACTTTCT TAGACATCTT TAATTAACAT GTTCTGGGTT
```

FIG. $A_{2\ cont.}$

```
4061  CAACAATACA AGGAAAAGTA TACATATAGG ACCAGGAAGA GCATTTTATA CAACAGGAGA AATAATAGGA
      GTTGTTATGT TCCTTTTCAT ATGTATATCC TGGTCCTTCT CGTAAAATAT GTTGTCCTCT TTATTATCCT

4131  GATATAAGAC AAGCACATTG CAACATTAGT AGAACAAAAT GGAATAACAC TTTAAATCAA ATAGCTACAA
      CTATATTCTG TTCGTGTAAC GTTGTAATCA TCTTGTTTTA CCTTATTGTG AAATTTAGTT TATCGATGTT

4201  AATTAAAAGA ACAATTTGGG AATAATAAAA CAATAGTCTT TAATCAATCC TCAGGAGGGG ACCCAGAAAT
      TTAATTTTCT TGTTAAACCC TTATTATTTT GTTATCAGAA ATTAGTTAGG AGTCCTCCCC TGGGTCTTTA

4271  TGTAATGCAC AGTTTTAATT GTGGAGGGGA ATTCTTCTAC TGTAATTCAA CACAACTGTT TAATAGTACT
      ACATTACGTG TCAAAATTAA CACCTCCCCT TAAGAAGATG ACATTAAGTT GTGTTGACAA ATTATCATGA

4341  TGGAATTTTA ATGGTACTTG GAATTTAACA CAATCGAATG GTACTGAAGG AAATGACACT ATCACACTCC
      ACCTTAAAAT TACCATGAAC CTTAAATTGT GTTAGCTTAC CATGACTTCC TTTACTGTGA TAGTGTGAGG

4411  CATGTAGAAT AAAACAAATT ATAAATATGT GGCAGGAAGT AGGAAAAGCA ATGTATGCCC CTCCCATCAG
      GTACATCTTA TTTTGTTTAA TATTTATACA CCGTCCTTCA TCCTTTTCGT TACATACGGG GAGGGTAGTC
```

FIG. $A_{2\ cont.}$

```
4481  AGGACAAATT AGATGCTCAT CAAATATTAC AGGGCTAATA TTAACAAGAG ATGGTGGAAC TAACAGTAGT
      TCCTGTTTAA TCTACGAGTA GTTTATAATG TCCCGATTAT AATTGTTCTC TACCACCTTG ATTGTCATCA

4551  GGGTCCGAGA TCTTCAGACC TGGGGGAGGA GATATGAGGG ACAATTGGAG AAGTGAATTA TATAAATATA
      CCCAGGCTCT AGAAGTCTGG ACCCCCTCCT CTATACTCCC TGTTAACCTC TTCACTTAAT ATATTTATAT

4621  AAGTAGTAAA AATTGAACCA TTAGGAGTAG CACCCACCAA GGCAAAAAGA AGAGTGGTGC AGAGAGAAAA
      TTCATCATTT TTAACTTGGT AATCCTCATC GTGGGTGGTT CCGTTTTTCT TCTCACCACG TCTCTCTTTT

4691  AAGAGCAGTG GGAACGATAG GAGCTATGTT CCTTGGGTTC TTGGGAGCAG CAGGAAGCAC TATGGGCGCA
      TTCTCGTCAC CCTTGCTATC CTCGATACAA GGAACCCAAG AACCCTCGTC GTCCTTCGTG ATACCCGCGT

4761  GCGTCAATAA CGCTGACGGT ACAGGCCAGA CTATTATTGT CTGGTATAGT GCAACAGCAG AACAATTTGC
      CGCAGTTATT GCGACTGCCA TGTCCGGTCT GATAATAACA GACCATATCA CGTTGTCGTC TTGTTAAACG

4831  TGAGGGCTAT TGAGGCGCAA CAGCATCTGT TGCAACTCAC AGTCTGGGGC ATCAAGCAGC TCCAGGCAAG
      ACTCCCGATA ACTCCGCGTT GTCGTAGACA ACGTTGAGTG TCAGACCCCG TAGTTCGTCG AGGTCCGTTC
```

FIG. $A_{2\ cont.}$

```
4901  AGTCCTGGCT GTGGAAAGAT ACCTAAGGGA TCAACAGCTC CTAGGGATTT GGGGTTGCTC TGGAAAACTC
      TCAGGACCGA CACCTTTCTA TGGATTCCCT AGTTGTCGAG GATCCCTAAA CCCCAACGAG ACCTTTTGAG

4971  ATCTGCACCA CTGCTGTGCC TTGGAATGCT AGTTGGAGTA ATAAAACTCT GGATATGATT TGGGATAACA
      TAGACGTGGT GACGACACGG AACCTTACGA TCAACCTCAT TATTTTGAGA CCTATACTAA ACCCTATTGT

5041  TGACCTGGAT GGAGTGGGAA AGAGAAATCG AAAATTACAC AGGCTTAATA TACACCTTAA TTGAGGAATC
      ACTGGACCTA CCTCACCCTT TCTCTTTAGC TTTTAATGTG TCCGAATTAT ATGTGGAATT AACTCCTTAG

5111  GCAGAACCAA CAAGAAAAGA ATGAACAAGA CTTATTAGCA TTAGATAAGT GGGCAAGTTT GTGGAATTGG
      CGTCTTGGTT GTTCTTTTCT TACTTGTTCT GAATAATCGT AATCTATTCA CCCGTTCAAA CACCTTAACC

5181  TTTGACATAT CAAATTGGCT GTGGTATGTA AAAATCTTCA TAATGATAGT AGGAGGCTTG ATAGGTTTAA
      AAACTGTATA GTTTAACCGA CACCATACAT TTTTAGAAGT ATTACTATCA TCCTCCGAAC TATCCAAATT

5251  GAATAGTTTT TACTGTACTT TCTATAGTAA ATAGAGTTAG GCAGGGATAC TCACCATTGT CATTTCAGAC
      CTTATCAAAA ATGACATGAA AGATATCATT TATCTCAATC CGTCCCTATG AGTGGTAACA GTAAAGTCTG
```

FIG. $A_{2\ cont.}$

```
5321  CCACCTCCCA GCCCCGAGGG GACCCGACAG GCCCGAAGGA ATCGAAGAAG AAGGTGGAGA CAGAGACTAA
      GGTGGAGGGT CGGGGCTCCC CTGGGCTGTC CGGGCTTCCT TAGCTTCTTC TTCCACCTCT GTCTCTGATT

5391  TTTTTATGCG GCCGCTGGTA CCCAACCTAA AAATTGAAAA TAAATACAAA GGTTCTTGAG GGTTGTGTTA
      AAAAATACGC CGGCGACCAT GGGTTGGATT TTTAACTTTT ATTTATGTTT CCAAGAACTC CCAACACAAT

5461  AATTGAAAGC GAGAAATAAT CATAAATAAG CCCGGGGATC CTCTAGAGTC GACACCATGG GTGCGAGAGC
      TTAACTTTCG CTCTTTATTA GTATTTATTC GGGCCCCTAG GAGATCTCAG CTGTGGTACC CACGCTCTCG

5531  GTCAGTATTA AGCGGGGGAG AATTAGATCG ATGGGAAAAA ATTCGGTTAA GGCCAGGGGG AAAGAAAAAA
      CAGTCATAAT TCGCCCCCTC TTAATCTAGC TACCCTTTTT TAAGCCAATT CCGGTCCCCC TTTCTTTTTT

5601  TATAAATTAA AACATATAGT ATGGGCAAGC AGGGAGCTAG AACGATTCGC AGTTAATCCT GGCCTGTTAG
      ATATTTAATT TTGTATATCA TACCCGTTCG TCCCTCGATC TTGCTAAGCG TCAATTAGGA CCGGACAATC

5671  AAACATCAGA AGGCTGTAGA CAAATACTGG GACAGCTACA ACCATCCCTT CAGACAGGAT CAGAAGAACT
      TTTGTAGTCT TCCGACATCT GTTTATGACC CTGTCGATGT TGGTAGGGAA GTCTGTCCTA GTCTTCTTGA
```

FIG. $A_{2\ cont.}$

```
5741  TAGATCATTA TATAATACAG TAGCAACCCT CTATTGTGTG CATCAAAGGA TAGAGATAAA AGACACCAAG
      ATCTAGTAAT ATATTATGTC ATCGTTGGGA GATAACACAC GTAGTTTCCT ATCTCTATTT TCTGTGGTTC

5811  GAAGCTTTAG ACAAGATAGA GGAAGAGCAA AACAAAAGTA AGAAAAAAGC ACAGCAAGCA GCAGCTGACA
      CTTCGAAATC TGTTCTATCT CCTTCTCGTT TTGTTTTCAT TCTTTTTTCG TGTCGTTCGT CGTCGACTGT

5881  CAGGACACAG CAATCAGGTC AGCCAAAATT ACCCTATAGT GCAGAACATC CAGGGGCAAA TGGTACATCA
      GTCCTGTGTC GTTAGTCCAG TCGGTTTTAA TGGGATATCA CGTCTTGTAG GTCCCCGTTT ACCATGTAGT

5951  GGCCATATCA CCTAGAACTT TAAATGCATG GGTAAAAGTA GTAGAAGAGA AGGCTTTCAG CCCAGAAGTG
      CCGGTATAGT GGATCTTGAA ATTTACGTAC CCATTTTCAT CATCTTCTCT TCCGAAAGTC GGGTCTTCAC

6021  ATACCCATGT TTTCAGCATT ATCAGAAGGA GCCACCCCAC AAGATTTAAA CACCATGCTA AACACAGTGG
      TATGGGTACA AAAGTCGTAA TAGTCTTCCT CGGTGGGGTG TTCTAAATTT GTGGTACGAT TTGTGTCACC

6091  GGGGACATCA AGCAGCCATG CAAATGTTAA AAGAGACCAT CAATGAGGAA GCTGCAGAAT GGGATAGAGT
      CCCCTGTAGT TCGTCGGTAC GTTTACAATT TTCTCTGGTA GTTACTCCTT CGACGTCTTA CCCTATCTCA
```

FIG. $A_{2\ cont.}$

```
6161  GCATCCAGTG CATGCAGGGC CTATTGCACC AGGCCAGATG AGAGAACCAA GGGGAAGTGA CATAGCAGGA
      CGTAGGTCAC GTACGTCCCG GATAACGTGG TCCGGTCTAC TCTCTTGGTT CCCCTTCACT GTATCGTCCT

6231  ACTACTAGTA CCCTTCAGGA ACAAATAGGA TGGATGACAA ATAATCCACC TATCCCAGTA GGAGAAATTT
      TGATGATCAT GGGAAGTCCT TGTTTATCCT ACCTACTGTT TATTAGGTGG ATAGGGTCAT CCTCTTTAAA

6301  ATAAAAGATG GATAATCCTG GGATTAAATA AAATAGTAAG AATGTATAGC CCTACCAGCA TTCTGGACAT
      TATTTTCTAC CTATTAGGAC CCTAATTTAT TTTATCATTC TTACATATCG GGATGGTCGT AAGACCTGTA

6371  AAGACAAGGA CCAAAAGAAC CCTTTAGAGA CTATGTAGAC CGGTTCTATA AAACTCTAAG AGCCGAGCAA
      TTCTGTTCCT GGTTTTCTTG CGAAATCTCT GATACATCTG GCCAAGATAT TTTGAGATTC TCGGCTCGTT

6441  GCTTCACAGG AGGTAAAAAA TTGGATGACA GAAACCTTGT TGGTCCAAAA TGCGAACCCA GATTGTAAGA
      CGAAGTGTCC TCCATTTTTT AACCTACTGT CTTTGGAACA ACCAGGTTTT ACGCTTGGGT CTAACATTCT

6511  CTATTTTAAA AGCATTGGGA CCAGCGGCTA CACTAGAAGA AATGATGACA GCATGTCAGG GAGTAGGAGG
      GATAAAATTT TCGTAACCCT GGTCGCCGAT GTGATCTTCT TTACTACTGT CGTACAGTCC CTCATCCTCC
```

FIG. $A_{2\ cont.}$

```
6581  ACCCGGCCAT AAGGCAAGAG TTTTGGCTGA AGCAATGAGC CAAGTAACAA ATTCAGCTAC CATAATGATG
      TGGGCCGGTA TTCCGTTCTC AAAACCGACT TCGTTACTCG GTTCATTGTT TAAGTCGATG GTATTACTAC

6651  CAGAGAGGCA ATTTTAGGAA CCAAAGAAAG ATTGTTAAGT GTTTCAATTG TGGCAAAGAA GGGCACACAG
      GTCTCTCCGT TAAAATCCTT GGTTTCTTTC TAACAATTCA CAAAGTTAAC ACCGTTTCTT CCCGTGTGTC

6721  CCAGAAATTG CAGGGCCCCT AGGAAAAAGG GCTGTTGGAA ATGTGGAAAG GAAGGACACC AAATGAAAGA
      GGTCTTTAAC GTCCCGGGGA TCCTTTTTCC CGACAACCTT TACACCTTTC CTTCCTGTGG TTTACTTTCT

6791  TTGTACTGAG AGACAGGCTA ATTTTTTAGG GAAGATCTGG CCTTCCTACA AGGGAAGGCC AGGGAATTTT
      AACATGACTC TCTGTCCGAT TAAAAAATCC CTTCTAGACC GGAAGGATGT TCCCTTCCGG TCCCTTAAAA

6861  CTTCAGAGCA GACCAGAGCC AACAGCCCCA CCAGAAGAGA GCTTCAGGTC TGGGGTAGAG ACAACAACTC
      GAAGTCTCGT CTGGTCTCGG TTGTCGGGGT GGTCTTCTCT CGAAGTCCAG ACCCCATCTC TGTTGTTGAG

6931  CCCCTCAGAA GCAGGAGCCG ATAGACAAGG AACTGTATCC TTTAACTTCC CTCAGATCAC TCTTTGGCAA
      GGGGAGTCTT CGTCCTCGGC TATCTGTTCC TTGACATAGG AAATTGAAGG GAGTCTAGTG AGAAACCGTT
```

FIG. $A_{2\ cont.}$

```
7001  CGACCCCTCG TCACAATAAA GATAGGGGGG CAACTAAAGG AAGCTCTATT AGATACAGGA GCAGATGATA
      GCTGGGGAGC AGTGTTATTT CTATCCCCCC GTTGATTTCC TTCGAGATAA TCTATGTCCT CGTCTACTAT

7071  CAGTATTAGA AGAAATGAGT TTGCCAGGAA GATGGAAACC AAAAATGATA GGGGGAATTG GAGGTTTTAT
      GTCATAATCT TCTTTACTCA AACGGTCCTT CTACCTTTGG TTTTTACTAT CCCCCTTAAC CTCCAAAATA

7141  CAAAGTAAGA CAGTATGATC AGATACTCAT AGAAATCTGT GGACATAAAG CTATAGGTAC AGTATTAGTA
      GTTTCATTCT GTCATACTAG TCTATGAGTA TCTTTAGACA CCTGTATTTC GATATCCATG TCATAATCAT

7211  GGACCTACAC CTGTCAACAT AATTGGAAGA AATCTGTTGA CTCAGATTGG TTGCACTTTA AATTTTCCCA
      CCTGGATGTG GACAGTTGTA TTAACCTTCT TTAGACAACT GAGTCTAACC AACGTGAAAT TTAAAAGGGT

7281  TTAGCCCTAT TGAGACTGTA CCAGTAAAAT TAAAGCCAGG AATGGATGGC CCAAAAGTTA AACAATGGCC
      AATCGGGATA ACTCTGACAT GGTCATTTTA ATTTCGGTCC TTACCTACCG GGTTTTCAAT TTGTTACCGG

7351  ATTGACAGAA GAAAAAATAA AAGCATTAGT AGAAATTTGT ACAGAAATGG AAAAGGAAGG GAAAATTTCA
      TAACTGTCTT CTTTTTTATT TTCGTAATCA TCTTTAAACA TGTCTTTACC TTTTCCTTCC CTTTTAAAGT
```

FIG. $A_{2\ cont.}$

```
7421  AAAATTGGGC CTGAGAATCC ATACAATACT CCAGTATTTG CCATAAAGAA AAAAGACAGT ACTAAATGGA
      TTTTAACCCG GACTCTTAGG TATGTTATGA GGTCATAAAC GGTATTTCTT TTTTCTGTCA TGATTTACCT

7491  GGAAATTAGT AGATTTCAGA GAACTTAATA AGAGAACTCA AGACTTCTGG GAAGTTCAAT TAGGAATACC
      CCTTTAATCA TCTAAAGTCT CTTGAATTAT TCTCTTGAGT TCTGAAGACC CTTCAAGTTA ATCCTTATGG

7561  ACATCCCGCA GGGTTAAAAA AGAAAAAATC AGTAACAGTA CTGGATGTGG GTGATGCATA TTTTTCAGTT
      TGTAGGGCGT CCCAATTTTT TCTTTTTTAG TCATTGTCAT GACCTACACC CACTACGTAT AAAAAGTCAA

7631  CCCTTAGATG AAGACTTCAG GAAGTATACT GCATTTACCA TACCTAGTAT AAACAATGAG ACACCAGGGA
      GGGAATCTAC TTCTGAAGTC CTTCATATGA CGTAAATGGT ATGGATCATA TTTGTTACTC TGTGGTCCCT

7701  TTAGATATCA GTACAATGTG CTTCCACAGG GATGGAAAGG ATCACCAGCA ATATTCCAAA GTAGCATGAC
      AATCTATAGT CATGTTACAC GAAGGTGTCC CTACCTTTCC TAGTGGTCGT TATAAGGTTT CATCGTACTG

7771  AAAAATCTTA GAGCCTTTTA AAAAACAAAA TCCAGACATA GTTATCTATC AATACATGAA CGATTTGTAT
      TTTTTAGAAT CTCGGAAAAT TTTTTGTTTT AGGTCTGTAT CAATAGATAG TTATGTACTT GCTAAACATA
```

FIG. $A_{2\ cont.}$

```
7841  GTAGGATCTG ACTTAGAAAT AGGGCAGCAT AGAACAAAAA TAGAGGAGCT GAGACAACAT CTGTTGAGGT
      CATCCTAGAC TGAATCTTTA TCCCGTCGTA TCTTGTTTTT ATCTCCTCGA CTCTGTTGTA GACAACTCCA

7911  GGGGACTTAC CACACCAGAC AAAAAACATC AGAAAGAACC TCCATTCCTT TGGATGGGTT ATGAACTCCA
      CCCCTGAATG GTGTGGTCTG TTTTTTGTAG TCTTTCTTGG AGGTAAGGAA ACCTACCCAA TACTTGAGGT

7981  TCCTGATAAA TGGACAGTAC AGCCTATAGT GCTGCCAGAA AAAGACAGCT GGACTGTCAA TGACATACAG
      AGGACTATTT ACCTGTCATG TCGGATATCA CGACGGTCTT TTTCTGTCGA CCTGACAGTT ACTGTATGTC

8051  AAGTTAGTGG GGAAATTGAA TACCGCAAGT CAGATTTACC CAGGGATTAA AGTAAGGCAA TTATGTAAAC
      TTCAATCACC CCTTTAACTT ATGGCGTTCA GTCTAAATGG GTCCCTAATT TCATTCCGTT AATACATTTG

8121  TCCTTAGAGG AACCAAAGCA CTAACAGAAG TAATACCACT AACAGAAGAA GCAGAGCTAG AACTGGCAGA
      AGGAATCTCC TTGGTTTCGT GATTGTCTTC ATTATGGTGA TTGTCTTCTT CGTCTCGATC TTGACCGTCT

8191  AAACAGAGAG ATTCTAAAAG AACCAGTACA TGGAGTGTAT TATGACCCAT CAAAAGACTT AATAGCAGAA
      TTTGTCTCTC TAAGATTTTC TTGGTCATGT ACCTCACATA ATACTGGGTA GTTTTCTGAA TTATCGTCTT
```

FIG. $A_{2\ cont.}$

```
8261  ATACAGAAGC AGGGGCAAGG CCAATGGACA TATCAAATTT ATCAAGAGCC ATTTAAAAAT CTGAAAACAG
      TATGTCTTCG TCCCCGTTCC GGTTACCTGT ATAGTTTAAA TAGTTCTCGG TAAATTTTTA GACTTTTGTC

8331  GAAAATATGC AAGAATGAGG GGTGCCCACA CTAATGATGT AAAACAATTA ACAGAGGCAG TGCAAAAAAT
      CTTTTATACG TTCTTACTCC CCACGGGTGT GATTACTACA TTTTGTTAAT TGTCTCCGTC ACGTTTTTTA

8401  AACCACAGAA AGCATAGTAA TATGGGGAAA GACTCCTAAA TTTAAACTAC CCATACAAAA GGAAACATGG
      TTGGTGTCTT TCGTATCATT ATACCCCTTT CTGAGGATTT AAATTTGATG GGTATGTTTT CCTTTGTACC

8471  GAAACATGGT GGACAGAGTA TTGGCAAGCC ACCTGGATTC CTGAGTGGGA GTTTGTTAAT ACCCCTCCTT
      CTTTGTACCA CCTGTCTCAT AACCGTTCGG TGGACCTAAG GACTCACCCT CAAACAATTA TGGGGAGGAA

8541  TAGTGAAATT ATGGTACCAG TTAGAGAAAG AACCCATAGT AGGAGCAGAA ACCTTCTATG TAGATGGGGC
      ATCACTTTAA TACCATGGTC AATCTCTTTC TTGGGTATCA TCCTCGTCTT TGGAAGATAC ATCTACCCCG

8611  AGCTAACAGG GAGACTAAAT TAGGAAAAGC AGGATATGTT ACTAACAAAG GAAGACAAAA GGTTGTCCCC
      TCGATTGTCC CTCTGATTTA ATCCTTTTCG TCCTATACAA TGATTGTTTC CTTCTGTTTT CCAACAGGGG
```

FIG. $A_{2\ cont.}$

8681 CTAACTAACA CAACAAATCA GAAAACTCAG TTACAAGCAA TTTATCTAGC TTTGCAGGAT TCAGGATTAG
GATTGATTGT GTTGTTTAGT CTTTTGAGTC AATGTTCGTT AAATAGATCG AAACGTCCTA AGTCCTAATC

8751 AAGTAAACAT AGTAACAGAC TCACAATATG CATTAGGAAT CATTCAAGCA CAACCAGATA AAAGTGAATC
TTCATTTGTA TCATTGTCTG AGTGTTATAC GTAATCCTTA GTAAGTTCGT GTTGGTCTAT TTTCACTTAG

8821 AGAGTTAGTC AATCAAATAA TAGAGCAGTT AATAAAAAAG GAAAAGGTCT ATCTGGCATG GGTACCAGCA
TCTCAATCAG TTAGTTTATT ATCTCGTCAA TTATTTTTTC CTTTTCCAGA TAGACCGTAC CCATGGTCGT

8891 CACAAAGGAA TTGGAGGAAA TGAACAAGTA GATAAATTAG TCAGTGCTGG AATCAGGAAA ATACTATTTT
GTGTTTCCTT AACCTCCTTT ACTTGTTCAT CTATTTAATC AGTCACGACC TTAGTCCTTT TATGATAAAA

8961 TAGATGGAAT AGATAAGGCC CAAGATGAAC ATTAGTTTTT ATGTCGACCT GCAGGGAAAG TTTTATAGGT
ATCTACCTTA TCTATTCCGG GTTCTACTTG TAATCAAAAA TACAGCTGGA CGTCCCTTTC AAAATATCCA

9031 AGTTGATAGA ACAAAATACA TAATTTTGTA AAAATAAATC ACTTTTTATA CTAATATGAC ACGATTACCA
TCAACTATCT TGTTTTATGT ATTAAAACAT TTTTATTTAG TGAAAAATAT GATTATACTG TGCTAATGGT

FIG. $A_{2\ cont.}$

9101 ATACTTTTGT TACTAATATC ATTAGTATAC GCTACACCTT TTCCTCAGAC ATCTAAAAAA ATAGGTGATG
TATGAAAACA ATGATTATAG TAATCATATG CGATGTGGAA AAGGAGTCTG TAGATTTTTT TATCCACTAC

9171 ATGCAACTTT ATCATGTAAT CGAAATAATA CAAATGACTA CGTTGTTATG AGTGCTTGGT ATAAGGAGCC
TACGTTGAAA TAGTACATTA GCTTTATTAT GTTTACTGAT GCAACAATAC TCACGAACCA TATTCCTCGG

9241 CAATTCCATT ATTCTTTTAG CTGCTAAAAG CGACGTCTTG TATTTTGATA ATTATACCAA GGATAAAATA
GTTAAGGTAA TAAGAAAATC GACGATTTTC GCTGCAGAAC ATAAAACTAT TAATATGGTT CCTATTTTAT

9311 TCTTACGACT CTCCATACGA TGATCTAGTT ACAACTATCA CAATTAAATC ATTGACTGCT AGAGATGCCG
AGAATGCTGA GAGGTATGCT ACTAGATCAA TGTTGATAGT GTTAATTTAG TAACTGACGA TCTCTACGGC

9381 GTACTTATGT ATGTGCATTC TTTATGACAT CGCCTACAAA TGACACTGAT AAAGTAGATT ATGAAGAATA
CATGAATACA TACACGTAAG AAATACTGTA GCGGATGTTT ACTGTGACTA TTTCATCTAA TACTTCTTAT

9451 CTCCACAGAG TTGATTGTAA ATACAGATAG TGAATCGACT ATAGACATAA TACTATCTGG ATCTACACAT
GAGGTGTCTC AACTAACATT TATGTCTATC ACTTAGCTGA TATCTGTATT ATGATAGACC TAGATGTGTA

FIG. $A_{2\ cont.}$

```
9521  TCACCAGAAA CTAGTTAAGC TTGTCTCCCT ATAGTGAGTC GTATTAGAGC TTGGCGTAAT CATGGTCATA
      AGTGGTCTTT GATCAATTCG AACAGAGGGA TATCACTCAG CATAATCTCG AACCGCATTA GTACCAGTAT

9591  GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT
      CGACAAAGGA CACACTTTAA CAATAGGCGA GTGTTAAGGT GTGTTGTATG CTCGGCCTTC GTATTTCACA

9661  AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCGAGT
      TTTCGGACCC CACGGATTAC TCACTCGATT GAGTGTAATT AACGCAACGC GAGTGACGGG CGAAAGCTCA

9731  CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGCGTATTGG
      GCCCTTTGGA CAGCACGGTC GACGTAATTA CTTAGCCGGT TGCGCGCCCC TCTCCGCCAA ACGCATAACC

9801  GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT
      CGCGAGAAGG CGAAGGACCG AGTGACTGAG CGACGCGAGC CAGCAAGCCG ACGCCGCTCG CCATAGTCGA

9871  CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG
      GTGAGTTTCC GCCATTATGC CAATAGGTGT CTTAGTCCCC TATTGCGTCC TTTCTTGTAC ACTCGTTTTC

9941  GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC GATAGGCTCC GCCCCCCTGA
      CGGTCGTTTT CCGGTCCTTG GCATTTTTCC GGCGCAACGA CCGCAAAAAG CTATCCGAGG CGGGGGGACT
```

FIG. $A_{2\ cont.}$

```
10011  CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG
       GCTCGTAGTG TTTTTAGCTG CGAGTTCAGT CTCCACCGCT TTGGGCTGTC CTGATATTTC TATGGTCCGC

10081  TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT
       AAAGGGGGAC CTTCGAGGGA GCACGCGAGA GGACAAGGCT GGGACGGCGA ATGGCCTATG GACAGGCGGA

10151  TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT
       AAGAGGGAAG CCCTTCGCAC CGCGAAAGAG TATCGAGTGC GACATCCATA GAGTCAAGCC ACATCCAGCA

10221  TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT
       AGCGAGGTTC GACCCGACAC ACGTGCTTGG GGGGCAAGTC GGGCTGGCGA CGCGGAATAG GCCATTGATA

10291  CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA
       GCAGAACTCA GGTTGGGCCA TTCTGTGCTG AATAGCGGTG ACCGTCGTCG GTGACCATTG TCCTAATCGT

10361  GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC
       CTCGCTCCAT ACATCCGCCA CGATGTCTCA AGAACTTCAC CACCGGATTG ATGCCGATGT GATCTTCCTG

10431  AGTATTTGGT ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC
       TCATAAACCA TAGACGCGAG ACGACTTCGG TCAATGGAAG CCTTTTTCTC AACCATCGAG AACTAGGCCG
```

FIG. $A_{2\ cont.}$

```
10501  AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT
       TTTGTTTGGT GGCGACCATC GCCACCAAAA AAACAAACGT TCGTCGTCTA ATGCGCGTCT TTTTTTCCTA

10571  CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT
       GAGTTCTTCT AGGAAACTAG AAAAGATGCC CCAGACTGCG AGTCACCTTG CTTTTGAGTG CAATTCCCTA

10641  TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA
       AAACCAGTAC TCTAATAGTT TTTCCTAGAA GTGGATCTAG GAAAATTTAA TTTTTACTTC AAAATTTAGT

10711  ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG
       TAGATTTCAT ATATACTCAT TTGAACCAGA CTGTCAATGG TTACGAATTA GTCACTCCGT GGATAGAGTC

10781  CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG
       GCTAGACAGA TAAAGCAAGT AGGTATCAAC GGACTGAGGG GCAGCACATC TATTGATGCT ATGCCCTCCC

10851  CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA
       GAATGGTAGA CCGGGGTCAC GACGTTACTA TGGCGCTCTG GGTGCGAGTG GCCGAGGTCT AAATAGTCGT

10921  ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA
       TATTTGGTCG GTCGGCCTTC CCGGCTCGCG TCTTCACCAG GACGTTGAAA TAGGCGGAGG TAGGTCAGAT
```

FIG. $A_{2\ cont.}$

```
10991  TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGGCATTGC
       AATTAACAAC GGCCCTTCGA TCTCATTCAT CAAGCGGTCA ATTATCAAAC GCGTTGCAAC AACCGTAACG

11061  TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG
       ATGTCCGTAG CACCACAGTG CGAGCAGCAA ACCATACCGA AGTAAGTCGA GGCCAAGGGT TGCTAGTTCC

11131  CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA
       GCTCAATGTA CTAGGGGGTA CAACACGTTT TTTCGCCAAT CGAGGAAGCC AGGAGGCTAG CAACAGTCTT

11201  GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC
       CATTCAACCG GCGTCACAAT AGTGAGTACC AATACCGTCG TGACGTATTA AGAGAATGAC AGTACGGTAG

11271  CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG
       GCATTCTACG AAAAGACACT GACCACTCAT GAGTTGGTTC AGTAAGACTC TTATCACATA CGCCGCTGGC

11341  AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA
       TCAACGAGAA CGGGCCGCAG TTATGCCCTA TTATGGCGCG GTGTATCGTC TTGAAATTTT CACGAGTAGT
```

FIG. $A_{2\ cont.}$

```
11411  TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC
       AACCTTTTGC AAGAAGCCCC GCTTTTGAGA GTTCCTAGAA TGGCGACAAC TCTAGGTCAA GCTACATTGG

11481  CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA
       GTGAGCACGT GGGTTGACTA GAAGTCGTAG AAAATGAAAG TGGTCGCAAA GACCCACTCG TTTTTGTCCT

11551  AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC
       TCCGTTTTAC GGCGTTTTTT CCCTTATTCC CGCTGTGCCT TTACAACTTA TGAGTATGAG AAGGAAAAAG

11621  AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA
       TTATAATAAC TTCGTAAATA GTCCCAATAA CAGAGTACTC GCCTATGTAT AAACTTACAT AAATCTTTTT

11691  TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC
       ATTTGTTTAT CCCCAAGGCG CGTGTAAAGG GGCTTTTCAC GGTGGACTGC AGATTCTTTG GTAATAATAG

11761  ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTCGC GCGTTTCGGT GATGACGGTG
       TACTGTAATT GGATATTTTT ATCCGCATAG TGCTCCGGGA AAGCAGAGCG CGCAAAGCCA CTACTGCCAC
```

FIG. $A_{2\ cont.}$

```
11831  AAAACCTCTG ACACATGCAG CTCCCGGAGA CGGTCACAGC TTGTCTGTAA GCGGATGCCG GGAGCAGACA
       TTTTGGAGAC TGTGTACGTC GAGGGCCTCT GCCAGTGTCG AACAGACATT CGCCTACGGC CCTCGTCTGT

11901  AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG CGGGTGTCGG GGCTGGCTTA ACTATGCGGC ATCAGAGCAG
       TCGGGCAGTC CCGCGCAGTC GCCCACAACC GCCCACAGCC CCGACCGAAT TGATACGCCG TAGTCTCGTC

11971  ATTGTACTGA GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA
       TAACATGACT CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT ATGGCGTAGT

12041  GGCGCCATTC GCCATTCAGG CTGCGCAACT GTTGGGAAGG GCGATCGGTG CGGGCCTCTT CGCTATTACG
       CCGCGGTAAG CGGTAAGTCC GACGCGTTGA CAACCCTTCC CGCTAGCCAC GCCCGGAGAA GCGATAATGC

12111  CCAGCTGGCG AAAGGGGGAT GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA
       GGTCGACCGC TTTCCCCCTA CACGACGTTC CGCTAATTCA ACCCATTGCG GTCCCAAAAG GGTCAGTGCT

12181  CGTTGTAAAA CGACGGCCAG TGAATTGGAT TTAGGTGACA CTATA
       GCAACATTTT GCTGCCGGTC ACTTAACCTA AATCCACTGT GATAT
```

FIG. $A_{2\ cont.}$

Text File of pLW-48 and the Included Individual HIV Genes and Their Promoters

Entire pLW-48 plasmid sequence:

GAATTCGTTGGTGGTCGCCATGGATGGTGTTATTGTATACTGTCTAAACGCG
TTAGTAAAACATGGCGAGGAAATAAATCATATAAAAAATGATTTCATGATTAA
ACCATGTTGTGAAAAAGTCAAGAACGTTCACATTGGCGGACAATCTAAAAAC
AATACAGTGATTGCAGATTTGCCATATATGGATAATGCGGTATCCGATGTAT
GCAATTCACTGTATAAAAAGAATGTATCAAGAATATCCAGATTTGCTAATTTG
ATAAAGATAGATGACGATGACAAGACTCCTACTGGTGTATATAATTATTTTAA
ACCTAAAGATGCCATTCCTGTTATTATATCCATAGGAAAGGATAGAGATGTTT
GTGAACTATTAATCTCATCTGATAAAGCGTGTGCGTGTATAGAGTTAAATTCA
TATAAAGTAGCCATTCTTCCCATGGATGTTTCCTTTTTTACCAAAGGAAATGC
ATCATTGATTATTCTCCTGTTTGATTTCTCTATCGATGCGGCACCTCTCTTAA
GAAGTGTAACCGATAATAATGTTATTATATCTAGACACCAGCGTCTACATGA
CGAGCTTCCGAGTTCCAATTGGTTCAAGTTTTACATAAGTATAAAGTCCGAC
TATTGTTCTATATTATATATGGTTGTTGATGGATCTGTGATGCATGCAATAGC
TGATAATAGAACTTACGCAAATATTAGCAAAAATATATTAGACAATACTACAA
TTAACGATGAGTGTAGATGCTGTTATTTTGAACCACAGATTAGGATTCTTGAT
AGAGATGAGATGCTCAATGGATCATCGTGTGATATGAACAGACATTGTATTA
TGATGAATTTACCTGATGTAGGCGAATTTGGATCTAGTATGTTGGGGAAATA
TGAACCTGACATGATTAAGATTGCTCTTTCGGTGGCTGGGTACCAGGCGCG
CCTTTCATTTTGTTTTTTTCTATGCTATAAATGGTACGTCCTGTAGAAACCCC
AACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCG
CGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAG
CCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCAGA
TATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCG
AAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCAT
TACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCAGGGCGG
CTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAG
TGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCC
GCCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTT
CCATGATTTCTTTAACTATGCCGGAATCCATCGCAGCGTAATGCTCTACACC
ACGCCGAACACCTGGGTGGACGATATCACCGTGGTGACGCATGTCGCGCA
AGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT
CAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAG
GCACTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGT
GAAGGTTATCTCTATGAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGT
GATATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGA
ACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCAT
GAAGATGCGGACTTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCAC
GACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGCAT
TACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTG

Figure B_1

GTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTT
TCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAAC
GGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGT
GACAAAAACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGAT
ACCCGTCCGCAAGGTGCACGGGAATATTTCGCGCCACTGGCGGAAGCAAC
GCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTG
CGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAA
CCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAA
GGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGAT
TATCATCACCGAATACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTA
CACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATCA
CCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTT
CGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAA
AGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCA
AAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGGCA
AACAATGAGAGCTCGGTTGTTGATGGATCTGTGATGCATGCAATAGCTGATA
ATAGAACTTACGCAAATATTAGCAAAAATATATTAGACAATACTACAATTAAC
GATGAGTGTAGATGCTGTTATTTTGAACCACAGATTAGGATTCTTGATAGAG
ATGAGATGCTCAATGGATCATCGTGTGATATGAACAGACATTGTATTATGAT
GAATTTACCTGATGTAGGCGAATTTGGATCTAGTATGTTGGGGAAATATGAA
CCTGACATGATTAAGATTGCTCTTTCGGTGGCTGGCGGCCCGCTCGAGTAA
AAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCTAT
GCTATAAATAATAAATAGCGGCCGCACCATGAAAGTGAAGGGGATCAGGAA
GAATTATCAGCACTTGTGGAAATGGGGCATCATGCTCCTTGGGATGTTGATG
ATCTGTAGTGCTGTAGAAAATTTGTGGGTCACAGTTTATTATGGGGTACCTG
TGTGGAAAGAAGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATA
TGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGA
CCCCAACCCACAAGAAGTAGTATTGGAAAATGTGACAGAAAATTTTAACATG
TGGAAAAATAACATGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGG
ATCAAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTACTTTAAAT
TGCACTGATTTGAGGAATGTTACTAATATCAATAATAGTAGTGAGGGAATGA
GAGGAGAAATAAAAAACTGCTCTTTCAATATCACCACAAGCATAAGAGATAA
GGTGAAGAAAGACTATGCACTTTTcTATAGACTTGATGTAGTACCAATAGATA
ATGATAATACTAGCTATAGGTTGATAAATTGTAATACCTCAACCATTACACAG
GCCTGTCCAAAGGTATCCTTTGAGCCAATTCCCATACATTATTGTACCCCGG
CTGGTTTTGCGATTCTAAAGTGTAAAGACAAGAAGTTCAATGGAACAGGGCC
ATGTAAAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCAGTAGTG
TCAACTCAACTGCTGTTAAATGGCAGTCTAGCAGAAGAAGAGGTAGTAATTA
GATCTAGTAATTTCACAGACAATGCAAAAAACATAATAGTACAGTTGAAAGAA
TCTGTAGAAATTAATTGTACAAGACCCAACAACAATACAAGGAAAAGTATAC
ATATAGGACCAGGAAGAGCATTTTATACAACAGGAGAAATAATAGGAGATAT
AAGACAAGCACATTGCAACATTAGTAGAACAAAATGGAATAACACTTTAAAT
CAAATAGCTACAAAATTAAAAGAACAATTTGGGAATAATAAAACAATAGTCTT
TAATCAATCCTCAGGAGGGGACCCAGAAATTGTAATGCACAGTTTTAATTGT
GGAGGGGAATTCTTCTACTGTAATTCAACACAACTGTTTAATAGTACTTGGA
ATTTTAATGGTACTTGGAATTTAACACAATCGAATGGTACTGAAGGAAATGA

Figure B_2

CACTATCACACTCCCATGTAGAATAAAACAAATTATAAATATGTGGCAGGAA
GTAGGAAAAGCAATGTATGCCCCTCCCATCAGAGGACAAATTAGATGCTCAT
CAAATATTACAGGGCTAATATTAACAAGAGATGGTGGAACTAACAGTAGTGG
GTCCGAGATCTTCAGACCTGGGGGAGGAGATATGAGGGACAATTGGAGAA
GTGAATTATATAAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCC
ACCAAGGCAAAAAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAAC
GATAGGAGCTATGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGG
CGCAGCGTCAATAACGCTGACGGTACAGGCCAGACTATTATTGTCTGGTAT
AGTGCAACAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCT
GTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAGTCCTGG
CTGTGGAAAGATACCTAAGGGATCAACAGCTCCTAGGGATTTGGGGTTGCT
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AGTTTGTGGAATTGGTTTGACATATCAAATTGGCTGTGGTATGTAAAAATCTT
CATAATGATAGTAGGAGGCTTGATAGGTTTAAGAATAGTTTTTACTGTACTTT
CTATAGTAAATAGAGTTAGGCAGGGATACTCACCATTGTCATTTCAGACCCA
CCTCCCAGCCCCGAGGGGACCCGACAGGCCCGAAGGAATCGAAGAAGAAG
GTGGAGACAGAGACTAATTTTTATGCGGCCGCTGGTACCCAACCTAAAAATT
GAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAAT
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GCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGAAAAAATTCGGTTA
AGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGCA
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AGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGG
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GATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCA
GGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAAATT
TATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCC
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GTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTA
AAAAATTGGATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTA
AGACTATTTTAAAAGCATTGGGACCAGCGGCTACACTAGAAGAAATGATGAC
AGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTG
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Figure B_3

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TAGAGACAACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACTGT
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Figure B_4

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CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGG
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TTCGAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
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CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
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CTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT
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GAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT
CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC

Figure B_5

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CAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA
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TGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTA
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GTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC
GTTGTAAAACGACGGCCAGTGAATTGGATTTAGGTGACACTATA

New Psyn II Promoter which controls ADA envelope expression:

TAAAAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTC
TATGCTATAAATAATAAATA

ADA envelope truncated:

ATGAAAGTGAAGGGGATCAGGAAGAATTATCAGCACTTGTGGAAATGGGGC
ATCATGCTCCTTGGGATGTTGATGATCTGTAGTGCTGTAGAAAATTTGTGGG
TCACAGTTTATTATGGGGTACCTGTGTGGAAAGAAGCAACCACCACTCTATT
TTGTGCATCAGATGCTAAAGCATATGATACAGAGGTACATAATGTTTGGGCC
ACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAGTAGTATTGGAA
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ATGAGGATATAATCAGTTTATGGGATCAAAGCCTAAAGCCATGTGTAAAATT
AACCCCACTCTGTGTTACTTTAAATTGCACTGATTTGAGGAATGTTACTAATA
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TTGTAATACCTCAACCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCA
ATTCCCATACATTATTGTACCCCGGCTGGTTTTGCGATTCTAAAGTGTAAAG
ACAAGAAGTTCAATGGAACAGGGCCATGTAAAAATGTCAGCACAGTACAAT
GTACACATGGAATTAGGCCAGTAGTGTCAACTCAACTGCTGTTAAATGGCAG
TCTAGCAGAAGAAGAGGTAGTAATTAGATCTAGTAATTTCACAGACAATGCA
AAAAACATAATAGTACAGTTGAAAGAATCTGTAGAAATTAATTGTACAAGACC
CAACAACAATACAAGGAAAAGTATACATATAGGACCAGGAAGAGCATTTTAT
ACAACAGGAGAAATAATAGGAGATATAAGACAAGCACATTGCAACATTAGTA
GAACAAAATGGAATAACACTTTAAATCAAATAGCTACAAAATTAAAAGAACAA
TTTGGGAATAATAAAACAATAGTCTTTAATCAATCCTCAGGAGGGGACCCAG
AAATTGTAATGCACAGTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTCA
ACACAACTGTTTAATAGTACTTGGAATTTTAATGGTACTTGGAATTTAACACA

Figure B_6

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CAAATTATAAATATGTGGCAGGAAGTAGGAAAAGCAATGTATGCCCCTCCCA
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GGCTGTGGTATGTAAAAATCTTCATAATGATAGTAGGAGGCTTGATAGGTTT
AAGAATAGTTTTTACTGTACTTTCTATAGTAAATAGAGTTAGGCAGGGATACT
CACCATTGTCATTTCAGACCCACCTCCCAGCCCCGAGGGGACCCGACAGG
CCCGAAGGAATCGAAGAAGAAGGTGGAGACAGAGAC

PmH5 promoter (which controls HXB2 gag pol expression):

AAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGC
GAGAAATAATCATAAATA

HXB2 gag pol (with safety mutations, Δ integrase):

ATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGA
AAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATA
GTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTA
GAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTT
CAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCT
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TGACACAGGACACAGCAATCAGGTCAGCCAAAATTACCCTATAGTGCAGAA
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Figure B_7

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Figure B_8

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CTATCTGGCATGGGTACCAGCACACAAAGGAATTGGAGGAAATGAACAAGT
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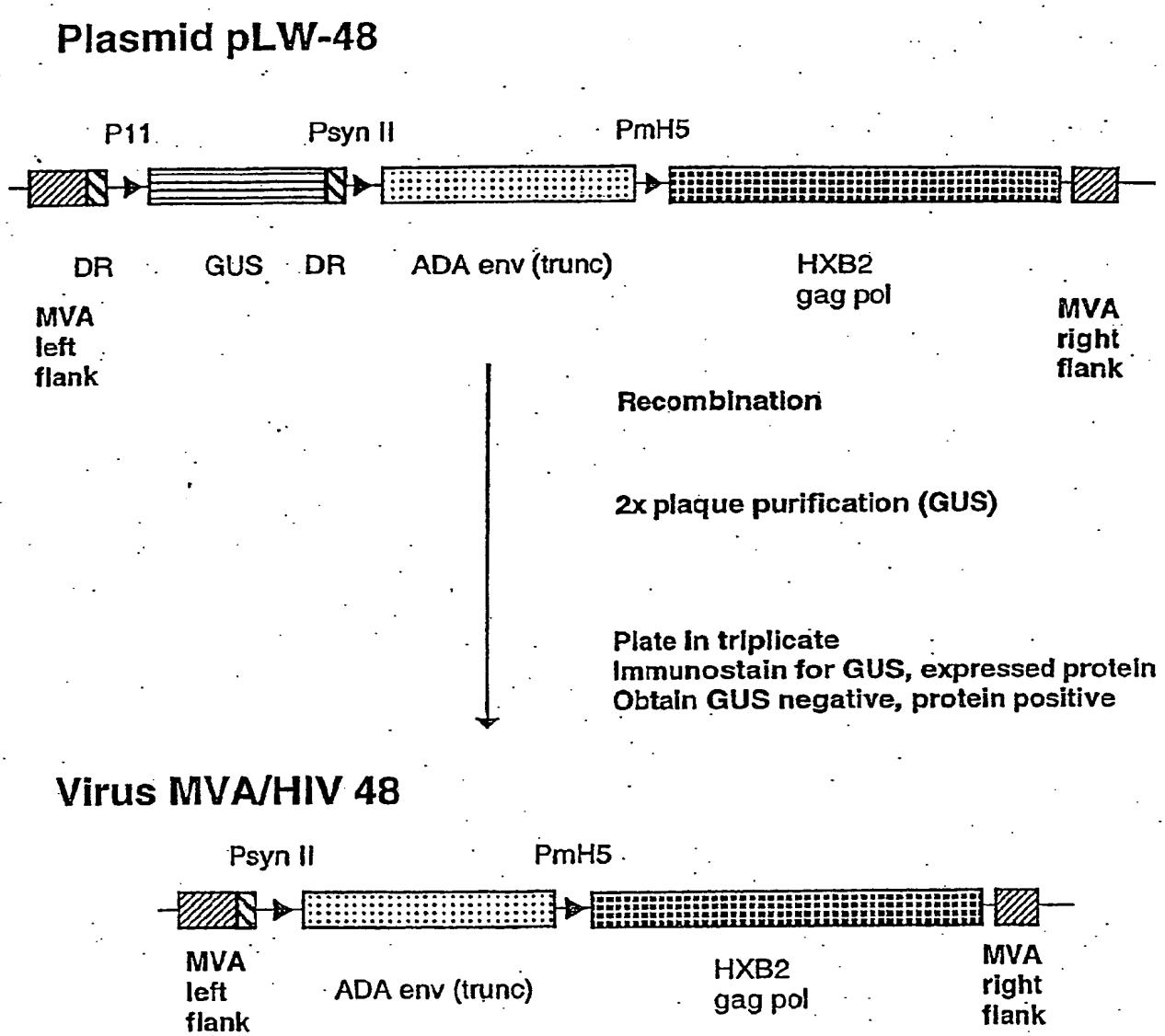
O:\DOCS\MXG\NIH211A.001PR\PLW-48 AND HIV GENES SEQ.DOC
022602

Figure B_9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PmH5
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GUS
DR
ADA env (trunc)
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flank
MVA
right
flank
Recombination
2x plaque purification (GUS)
Plate in triplicate
Immunostain for GUS, expressed protein
Obtain GUS negative, protein positive
Virus MVA/HIV 48
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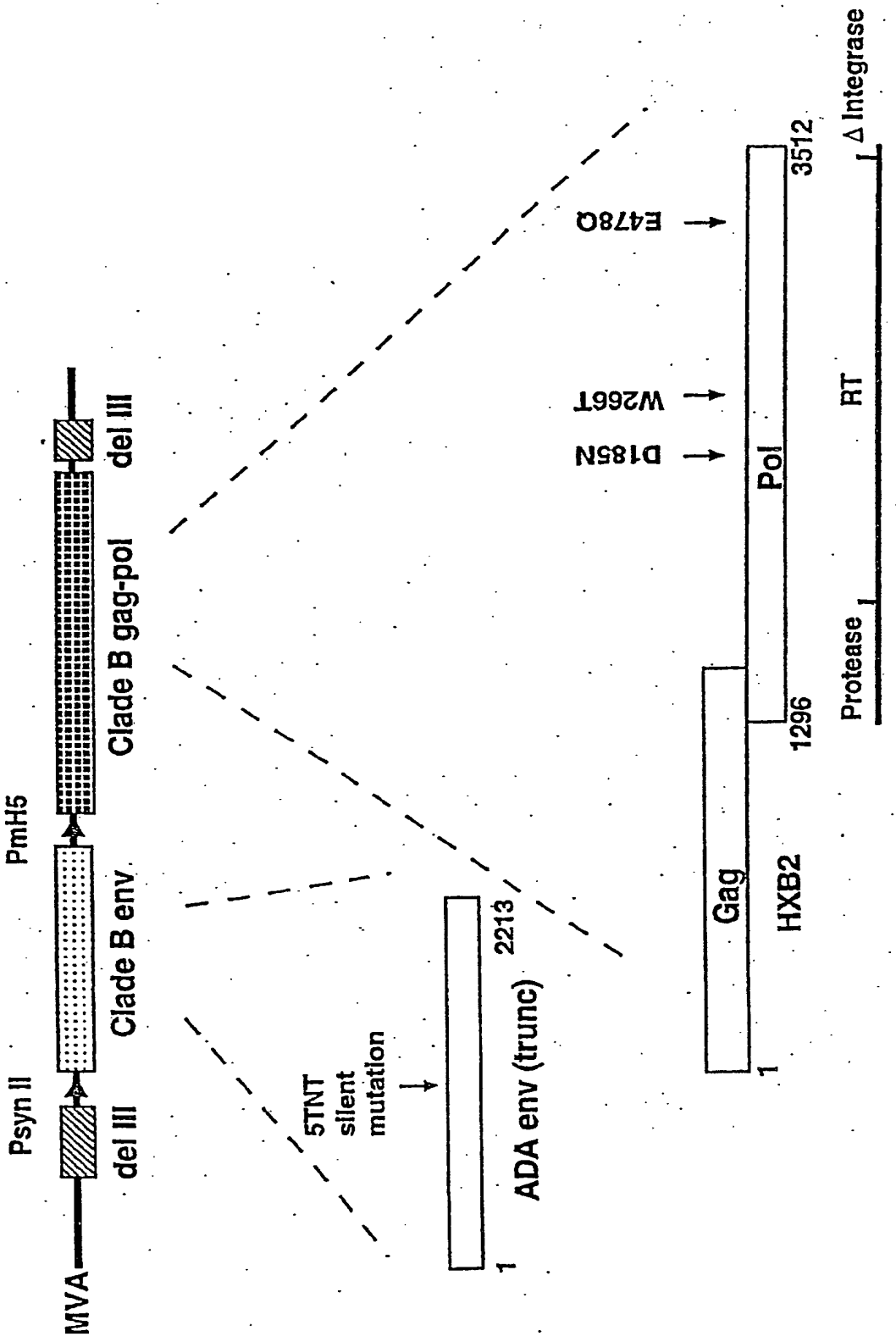
HXB2
gag pol
MVA
right
flank

Figure C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Psyn II
PmH5
Clade B env
Clade B gag-pol
del III
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mutation
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ADA env (trunc)
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1
Gag
HXB2
1296
Pol
3512
Protease
RT
Δ Integrase
D185N
W266T
E478Q

Figure D

Sequence of new Psyn II promoter:

Early part of promoter

Critical region Early start site

TAAAAAATGAAAAAATATTCTAATTTATAGGACGGT

Late part of promoter

TTTGATTTTCTTTTTTTCTATGCTATAAATAATAAATA

Figure E

SEQUENCE LISTING

<110> THE GOVERNMENT OF THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES
Moss, Bernard
Wyatt, Linda
Earl, Patricia

<120> MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

<130> NIH211.001PCT

<150> US 60/274,434
<151> 2001-03-08

<160> 13

<170> FastSEQ for Windows Version 4.0

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<211> 12225
<212> DNA
<213> Artificial Sequence

<220>
<223> Plasmid pLW-48

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agtcaagaac gttcacattg gcggacaatc taaaaacaat acagtgattg cagatttgcc 180
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(54) Title: MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

(57) Abstract: The invention provides modified virus Ankara (MVA), a replication-deficient strain of vaccinia virus, expressing human immunodeficiency virus (HIV) *env*, *gag*, and *pol* genes.

MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

Field of the Invention

The invention provides modified vaccinia Ankara (MVA), a replication-deficient strain of vaccinia virus, expressing human immunodeficiency virus (HIV) *env*, *gag*, and *pol* genes.

Background of the Invention

Cellular immunity plays an important role in the control of immunodeficiency virus infections (P.J. Goulder *et al.* 1999 *AIDS* **13**:S121). Recently, a DNA vaccine designed to enhance cellular immunity by cytokine augmentation successfully contained a highly virulent immunodeficiency virus challenge (D.H. Barouch *et al.* 2000 *Science* **290**:486). Another promising approach to raising cellular immunity is DNA priming followed by recombinant poxvirus boosters (H.L. Robinson *et al.* 2000 *AIDS Rev* **2**:105). This heterologous prime/boost regimen induces 10- to 100-fold higher frequencies of T cells than priming and boosting with DNA or recombinant poxvirus vaccines alone. Previously, investigators showed that boosting a DNA-primed response with a poxvirus was superior to boosting with DNA or protein for the control of a non-pathogenic immunodeficiency virus (H.L. Robinson *et al.* 1999 *Nat Med* **5**:526). There is a need for the control of a pathogenic immunodeficiency virus.

Summary of the Invention

Here we report that DNA priming followed by a recombinant modified vaccinia Ankara (rMVA) booster has controlled a highly pathogenic immunodeficiency virus challenge in a rhesus macaque model. Both the DNA and rMVA components of the vaccine expressed multiple immunodeficiency virus proteins. Two DNA inoculations at 0 and 8 weeks and a single rMVA booster at 24 weeks effectively controlled an intrarectal challenge administered seven months after the booster. These findings are envisioned as indicating that a relatively simple multiprotein DNA/MVA vaccine can help to control the acquired immune deficiency syndrome (AIDS) epidemic. We also report that inoculations of rMVA induce good immune responses even without DNA priming.

Brief Description of the Drawings

Figure I. Phylogenetic relationships of HIV-1 and HIV-2 based on identity of *pol* gene sequences. SIV_{cpz} and SIV_{smm} are subhuman primate lentiviruses recovered from a chimpanzee and sooty mangabey monkey, respectively.

Figure II. Phylogenetic relationships of HIV-1 groups M, N and O with four different SIV_{cpz} isolates based on full-length *pol* gene sequences. The bar indicates a genetic distance of 0.1 (10% nucleotide divergence) and the *asterisk* positions group N HIV-1 isolates based on *env* sequences.

Figure III. Tropic and biologic properties of HIV-1 isolates.

Figure IV. HIV-encoded proteins. The location of the HIV genes, the sizes of primary translation products (in some cases polyproteins), and the processed mature viral proteins are indicated.

Figure V. Schematic representation of a mature HIV-1 virion.

Figure VI. Linear representation of the HIV-1 Env glycoprotein. The *arrow* indicates the site of gp160 cleavage to gp120 and gp41. In gp120, *cross-hatched* areas represent variable domains (V_1 to V_5) and *open boxes* depict conserved sequences (C_1 to C_5). In the gp41 ectodomain, several domains are indicated: the N-terminal fusion peptide, and the two ectodomain helices (N- and C-helix). The membrane-spanning domain is represented by a *black box*. In the gp41 cytoplasmic domain, the Tyr-X-X-Leu (YXXL) endocytosis motif (SEQ ID NO: 9) and two predicted helical domains (helix-1 and –2) are shown. Amino acid numbers are indicated.

Figure 1. Temporal frequencies of Gag-specific T cells. (**A**) Gag-specific CD8 T cell responses raised by DNA priming and rMVA booster immunizations. The schematic presents mean Gag-CM9-tetramer data generated in the high-dose i.d. DNA-immunized animals. (**B**) Gag–specific IFN-γ ELISPOTs in *A*01* (open bars) and non-*A*01* (filled bars) macaques at various times before challenge and at two weeks after challenge. Three pools of 10 to 13 Gag peptides (22-mers overlapping by 12) were used for the analyses. The numbers above data bars represent the arithmetic mean ± SD for the ELISPOTs within each group. The numbers at the top of the graphs designate individual animals. *, data not available; #, <20 ELISPOTs per $1x10^6$ peripheral blood mononuclear cells (PBMC). Temporal data for Gag-CM9-Mamu-A*01 tetramer-specific T cells can be found in Figure 6.

Figure 2. Temporal viral loads, CD4 counts, and survival after challenge of vaccinated and control animals. (**A**) Geometric mean viral loads and (**B**) geometric mean CD4 counts. (**C**) Survival curve for vaccinated and control animals. The dotted line represents all 24 vaccinated animals. (**D**) Viral loads and (**E**) CD4 counts for individual animals in the vaccine and control groups. The key to animal numbers is presented in (**E**). Assays for the first 12 weeks after challenge had a detection level of 1000 copies of RNA per milliliter of plasma. Animals with loads below 1000 were scored with a load of 500. For weeks 16 and 20, the detection level was 300 copies of RNA per milliliter. Animals with levels of virus below 300 were scored at 300.

Figure 3. Postchallenge T cell responses in vaccine and control groups. (**A**) Temporal tetramer$^+$ cells (dashed line) and viral loads (solid line). (**B**) Intracellular cytokine assays for IFN-γ production in response to stimulation with the Gag-CM9 peptide at two weeks after challenge. This *ex vivo* assay allows evaluation of the functional status of the peak postchallenge tetramer$^+$ cells displayed in Figure 1A. (**C**) Proliferation assay at 12 weeks after challenge. Gag-Pol-Env (open bars) and Gag-Pol (hatched bars) produced by transient transfections were used for stimulation. Supernatants from mock-transfected cultures served as control antigen. Stimulation indices are the growth of cultures in the presence of viral antigens divided by the growth of cultures in the presence of mock antigen.

Figure 4. Lymph node histomorphology at 12 weeks after challenge. (**A**) Typical lymph node from a vaccinated macaque showing evidence of follicular hyperplasia characterized by the presence of numerous secondary follicles with expanded germinal centers and discrete dark and light zones. (**B**) Typical lymph node from an infected control animal showing follicular depletion and paracortical lymphocellular atrophy. (**C**) A representative lymph node from an age-matched, uninfected macaque displaying nonreactive germinal centers. (**D**) The percentage of the total lymph node area occupied by germinal centers was measured to give a non-specific indicator of follicular hyperplasia. Data for uninfected controls are for four age-matched rhesus macaques.

Figure 5. Temporal antibody responses. Micrograms of total Gag (**A**) or Env (**B**) antibody were determined with ELISAs. The titers of neutralizing antibody for SHIV-89.6 (**C**) and SHIV-89.6P (**D**) were determined with MT-2 cell killing and neutral red staining (D.C. Montefiori *et al.* 1988 *J Clin Microbiol* **26**:231). Titers are the reciprocal of the

serum dilution giving 50% neutralization of the indicated viruses grown in human PBMC. Symbols for animals are the same as in Figure 2.

Figure 6. Gag-CM9-Mamu-A*01 tetramer-specific T cells in *Mamu-A*01* vaccinated and control macaques at various times before challenge and at two weeks after challenge. The number at the upper right corner of each plot represents the frequency of tetramer-specific CD8 T cells as a % of total CD8 T cells. The numbers above each column of FACS data designate individual animals.

Figure A. Map and sequence of plasmid transfer vector pLW-48.

Figure B. Sequences of plasmid transfer vector pLW-48, Psy II promoter (which controls ADA envelope expression), ADA envelope truncated, PmH5 promoter (which controls HXB2 gag pol expression), and HXB2 *gag pol* (with safety mutations, Δ integrase).

Figure C. Plasmid transfer vector pLW-48 and making MVA recombinant virus MVA/HIV 48.

Figure D. A clade B gag pol.

Figure E. Sequence of new Psyn II promoter.

Detailed Description of the Preferred Embodiment

Recombinant MVA Virus

Vaccinia virus, a member of the genus Orthopoxvirus in the family of Poxviridae, was used as live vaccine to immunize against the human smallpox disease. Successful worldwide vaccination with vaccinia virus culminated in the eradication of variola virus, the causative agent of the smallpox (The global eradication of smallpox. Final report of the global commission for the certification of smallpox eradication. History of Public Health, No. 4, Geneva: World Health Organization, 1980). Since that WHO declaration, vaccination has been universally discontinued except for people at high risk of poxvirus infections (e.g. laboratory workers).

More recently, vaccinia viruses have also been used to engineer viral vectors for recombinant gene expression and for the potential use as recombinant live vaccines (Mackett, M. *et al.* 1982 *PNAS USA* **79**:7415-7419; Smith, G.L. *et al.* 1984 Biotech Genet Engin Rev **2**:383-407). This entails DNA sequences (genes) which code for foreign antigens being introduced, with the aid of DNA recombination techniques, into the genome of the vaccinia viruses. If the gene is integrated at a site in the viral DNA which is non-

essential for the life cycle of the virus, it is possible for the newly produced recombinant vaccinia virus to be infectious, that is to say able to infect foreign cells and thus to express the integrated DNA sequence (EP Patent Applications No. 83,286 and No. 110,385). The recombinant vaccinia viruses prepared in this way can be used, on the one hand, as live vaccines for the prophylaxis of infectious diseases, on the other hand, for the preparation of heterologous proteins in eukaryotic cells.

For vector applications health risks would be lessened by the use of a highly attenuated vaccinia virus strain. Several such strains of vaccinia virus were especially developed to avoid undesired side effects of smallpox vaccination. Thus, the modified vaccinia Ankara (MVA) has been generated by long-term serial passages of the Ankara strain of vaccinia virus (CVA) on chicken embryo fibroblasts (for review see Mayr, A. *et al.* 1975 *Infection* **3**:6-14; Swiss Patent No. 568,392). The MVA virus is publicly available from American Type Culture Collection as ATCC No. VR-1508. MVA is distinguished by its great attenuation, that is to say by diminished virulence and ability to replicate in primate cells while maintaining good immunogenicity. The MVA virus has been analyzed to determine alterations in the genome relative to the parental CVA strain. Six major deletions of genomic DNA (deletion I, II, III, IV, V, and VI) totaling 31,000 base pairs have been identified (Meyer, H. *et al.* 1991 *J Gen Virol* **72**:1031-1038). The resulting MVA virus became severely host cell restricted to avian cells.

Furthermore, MVA is characterized by its extreme attenuation. When tested in a variety of animal models, MVA was proven to be avirulent even in immunosuppressed animals. More importantly, the excellent properties of the MVA strain have been demonstrated in extensive clinical trials (Mayr A. *et al.* 1978 Zentralbl Bakteriol [B] **167**:375-390; Stickl *et al.* 1974 Dtsch Med Wschr **99**:2386-2392). During these studies in over 120,000 humans, including high-risk patients, no side effects were associated with the use of MVA vaccine.

MVA replication in human cells was found to be blocked late in infection preventing the assembly to mature infectious virions. Nevertheless, MVA was able to express viral and recombinant genes at high levels even in non-permissive cells and was proposed to serve as an efficient and exceptionally safe gene expression vector (Sutter, G. and Moss, B. 1992 *PNAS USA* **89**:10847-10851). Additionally, novel vaccinia vector vaccines were established on the basis of MVA having foreign DNA sequences inserted at

the site of deletion III within the MVA genome (Sutter, G. *et al.* 1994 Vaccine **12**:1032-1040).

The recombinant MVA vaccinia viruses can be prepared as set out hereinafter. A DNA-construct which contains a DNA-sequence which codes for a foreign polypeptide flanked by MVA DNA sequences adjacent to a naturally occurring deletion, e.g. deletion III, or other non-essential sites, within the MVA genome, is introduced into cells infected with MVA, to allow homologous recombination. Once the DNA-construct has been introduced into the eukaryotic cell and the foreign DNA has recombined with the viral DNA, it is possible to isolate the desired recombinant vaccinia virus in a manner known per se, preferably with the aid of a marker. The DNA-construct to be inserted can be linear or circular. A plasmid or polymerase chain reaction product is preferred. The DNA-construct contains sequences flanking the left and the right side of a naturally occurring deletion, e.g. deletion III, within the MVA genome. The foreign DNA sequence is inserted between the sequences flanking the naturally occurring deletion. For the expression of a DNA sequence or gene, it is necessary for regulatory sequences, which are required for the transcription of the gene, to be present on the DNA. Such regulatory sequences (called promoters) are known to those skilled in the art, and include for example those of the vaccinia 11 kDa gene as are described in EP-A-198,328, and those of the 7.5 kDa gene (EP-A-110,385). The DNA-construct can be introduced into the MVA infected cells by transfection, for example by means of calcium phosphate precipitation (Graham *et al.* 1973 *Virol* **52**:456-467; Wigler *et al.* 1979 *Cell* **16**:777-785), by means of electroporation (Neumann *et al.* 1982 *EMBO J* **1**:841-845), by microinjection (Graessmann *et al.* 1983 *Meth Enzymol* **101**:482-492), by means of liposomes (Straubinger *et al.* 1983 *Meth Enzymol* **101**:512-527), by means of spheroplasts (Schaffner 1980 *PNAS USA* **77**:2163-2167) or by other methods known to those skilled in the art.

HIVs and Their Replication

The etiological agent of acquired immune deficiency syndrome (AIDS) is recognized to be a retrovirus exhibiting characteristics typical of the lentivirus genus, referred to as human immunodeficiency virus (HIV). The phylogenetic relationships of the human lentiviruses are shown in Figure I. HIV-2 is more closely related to SIV_{smm}, a virus isolated from sooty mangabey monkeys in the wild, than to HIV-1. It is currently believed

that HIV-2 represents a zoonotic transmission of SIV_{smm} to man. A series of lentiviral isolates from captive chimpanzees, designated SIV_{cpz}, are close genetic relatives of HIV-1.

The earliest phylogenetic analyses of HIV-1 isolates focused on samples from Europe/North America and Africa; discrete clusters of viruses were identified from these two areas of the world. Distinct genetic subtypes or clades of HIV-1 were subsequently defined and classified into three groups: M (major); O (outlier); and N (non-M or O) (Fig. II). The M group of HIV-1, which includes over 95% of the global virus isolates, consists of at least eight discrete clades (A, B, C, D, F, G, H, and J), based on the sequence of complete viral genomes. Members of HIV-1 group O have been recovered from individuals living in Cameroon, Gabon, and Equatorial Guinea; their genomes share less than 50% identity in nucleotide sequence with group M viruses. The more recently discovered group N HIV-I strains have been identified in infected Cameroonians, fail to react serologically in standard whole-virus enzyme-linked immunosorbent assay (ELISA), yet are readily detectable by conventional Western blot analysis.

Most current knowledge about HIV-1 genetic variation comes from studies of group M viruses of diverse geographic origin. Data collected during the past decade indicate that the HIV-1 population present within an infected individual can vary from 6% to 10% in nucleotide sequence. HIV-1 isolates within a clade may exhibit nucleotide distances of 15% in *gag* and up to 30% in gp120 coding sequences. Interclade genetic variation may range between 30% and 40% depending on the gene analyzed.

All of the HIV-1 group M subtypes can be found in Africa. Clade A viruses are genetically the most divergent and were the most common HIV-1 subtype in Africa early in the epidemic. With the rapid spread of HIV-1 to southern Africa during the mid to late 1990s, clade C viruses have become the dominant subtype and now account for 48% of HIV-1 infections worldwide. Clade B viruses, the most intensively studied HIV-1 subtype, remain the most prevalent isolates in Europe and North America.

High rates of genetic recombination are a hallmark of retroviruses. It was initially believed that simultaneous infections by genetically diverse virus strains were not likely to be established in individuals at risk for HIV-1. By 1995, however, it became apparent that a significant fraction of the HIV-1 group M global diversity included interclade viral recombinants. It is now appreciated that HIV-1 recombinants will be found in geographic areas such as Africa, South America, and Southeast Asia, where multiple HIV-1 subtypes

coexist and may account for more than 10% of circulating HIV-1 strains. Molecularly, the genomes of these recombinant viruses resemble patchwork mosaics, with juxtaposed diverse HIV-1 subtype segments, reflecting the multiple crossover events contributing to their generation. Most HIV-1 recombinants have arisen in Africa and a majority contain segments originally derived from clade A viruses. In Thailand, for example, the composition of the predominant circulating strain consists of a clade A *gag* plus *pol* gene segment and a clade E *env* gene. Because the clade E *env* gene in Thai HIV-1 strains is closely related to the clade E *env* present in virus isolates from the Central African Republic, it is believed that the original recombination event occurred in Africa, with the subsequent introduction of a descendent virus into Thailand. Interestingly, no full-length HIV-1 subtype E isolate (i.e., with subtype E *gag*, *pol*, and *env* genes) has been reported to date.

The discovery that α and β chemokine receptors function as coreceptors for virus fusion and entry into susceptible $CD4^+$ cells has led to a revised classification scheme for HIV-1 (Fig. III). Isolates can now be grouped on the basis of chemokine receptor utilization in fusion assays in which HIV-1 gp120 and $CD4^+$ coreceptor proteins are expressed in separate cells. As indicated in Figure III, HIV-1 isolates using the CXCR4 receptor (now designated X4 viruses) are usually T cell line (TCL)-tropic syncytium inducing (SI) strains, whereas those exclusively utilizing the CCR5 receptor (R5 viruses) are predominantly macrophage (M)-tropic and non-syncytium inducing (NSI). The dual-tropic R5/X4 strains, which may comprise the majority of patient isolates and exhibit a continuum of tropic phenotypes, are frequently SI.

As is the case for all replication-competent retroviruses, the three primary HIV-1 translation products, all encoding structural proteins, are initially synthesized as polyprotein precursors, which are subsequently processed by viral or cellular proteases into mature particle-associated proteins (Fig. IV). The 55-kd Gag precursor $Pr55^{Gag}$ is cleaved into the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. Autocatalysis of the 160-kd Gag-Pol polyprotein, $Pr160^{Gag\text{-}Pol}$, gives rise to the protease (PR), the heterodimeric reverse transcriptase (RT), and the integrase (IN) proteins, whereas proteolytic digestion by a cellular enzyme(s) converts the glycosylated 160-kd Env precursor gp160 to the gp120 surface (SU) and gp41 transmembrane (TM) cleavage products. The remaining six HIV-1-

encoded proteins (Vif, Vpr, Tat, Rev, Vpu, and Nef) are the primary translation products of spliced mRNAs.

Gag

The Gag proteins of HIV, like those of other retroviruses, are necessary and sufficient for the formation of noninfectious, virus-like particles. Retroviral Gag proteins are generally synthesized as polyprotein precursors; the HIV-1 Gag precursor has been named, based on its apparent molecular mass, $Pr55^{Gag}$. As noted previously, the mRNA for $Pr55^{Gag}$ is the unspliced 9.2-kb transcript (Fig. IV) that requires Rev for its expression in the cytoplasm. When the *pol* ORF is present, the viral protease (PR) cleaves $Pr55^{Gag}$ during or shortly after budding from the cell to generate the mature Gag proteins p17 (MA), p24 (CA), p7 (NC), and p6 (see Fig. IV). In the virion, MA is localized immediately inside the lipid bilayer of the viral envelope, CA forms the outer portion of the cone-shaped core structure in the center of the particle, and NC is present in the core in a ribonucleoprotein complex with the viral RNA genome (Fig. V).

The HIV $Pr55^{Gag}$ precursor oligomerizes following its translation and is targeted to the plasma membrane, where particles of sufficient size and density to be visible by EM are assembled. Formation of virus-like particles by $Pr55^{Gag}$ is a self-assembly process, with critical Gag-Gag interactions taking place between multiple domains along the Gag precursor. The assembly of virus-like particles does not require the participation of genomic RNA (although the presence of nucleic acid appears to be essential), *pol*-encoded enzymes, or Env glycoproteins, but the production of infectious virions requires the encapsidation of the viral RNA genome and the incorporation of the Env glycoproteins and the Gag-Pol polyprotein precursor $Pr160^{Gag\text{-}Pol}$.

Pol

Downstream of *gag* lies the most highly conserved region of the HIV genome, the *pol* gene, which encodes three enzymes: PR, RT, and IN (see Fig. IV). RT and IN are required, respectively, for reverse transcription of the viral RNA genome to a double-stranded DNA copy, and for the integration of the viral DNA into the host cell chromosome. PR plays a critical role late in the life cycle by mediating the production of mature, infectious virions. The *pol* gene products are derived by enzymatic cleavage of a 160-kd Gag-Pol fusion protein, referred to as $Pr160^{Gag\text{-}Pol}$. This fusion protein is produced by ribosomal frameshifting during translation of $Pr55^{Gag}$ (see Fig. IV). The frame-shifting

mechanism for Gag-Pol expression, also utilized by many other retroviruses, ensures that the *pol*-derived proteins are expressed at a low level, approximately 5% to 10% that of Gag. Like $Pr55^{Gag}$, the N-terminus of $Pr160^{Gag\text{-}Pol}$ is myristylated and targeted to the plasma membrane.

Protease

Early pulse-chase studies performed with avian retroviruses clearly indicated that retroviral Gag proteins are initially synthesized as polyprotein precursors that are cleaved to generate smaller products. Subsequent studies demonstrated that the processing function is provided by a viral rather than a cellular enzyme, and that proteolytic digestion of the Gag and Gag-Pol precursors is essential for virus infectivity. Sequence analysis of retroviral PRs indicated that they are related to cellular "aspartic" proteases such as pepsin and renin. Like these cellular enzymes, retroviral PRs use two apposed Asp residues at the active site to coordinate a water molecule that catalyzes the hydrolysis of a peptide bond in the target protein. Unlike the cellular aspartic proteases, which function as pseudodimers (using two folds within the same molecule to generate the active site), retroviral PRs function as true dimers. X-ray crystallographic data from HIV-1 PR indicate that the two monomers are held together in part by a four-stranded antiparallel β-sheet derived from both N- and C-terminal ends of each monomer. The substrate-binding site is located within a cleft formed between the two monomers. Like their cellular homologs, the HIV PR dimer contains flexible "flaps" that overhang the binding site and may stabilize the substrate within the cleft; the active-site Asp residues lie in the center of the dimer. Interestingly, although some limited amino acid homology is observed surrounding active-site residues, the primary sequences of retroviral PRs are highly divergent, yet their structures are remarkably similar.

Reverse Transcriptase

By definition, retroviruses possess the ability to convert their single-stranded RNA genomes into double-stranded DNA during the early stages of the infection process. The enzyme that catalyzes this reaction is RT, in conjunction with its associated RNaseH activity. Retroviral RTs have three enzymatic activities: (a) RNA-directed DNA polymerization (for minus-strand DNA synthesis), (b) RNaseH activity (for the degradation of the tRNA primer and genomic RNA present in DNA-RNA hybrid intermediates), and (c) DNA-directed DNA polymerization (for second- or plus-strand DNA synthesis).

The mature HIV-1 RT holoenzyme is a heterodimer of 66 and 51 kd subunits. The 51-kd subunit (p51) is derived from the 66-kd (p66) subunit by proteolytic removal of the C-terminal 15-kd RNaseH domain of p66 by PR (see Fig. IV). The crystal structure of HIV-1 RT reveals a highly asymmetric folding in which the orientations of the p66 and p51 subunits differ substantially. The p66 subunit can be visualized as a right hand, with the polymerase active site within the palm, and a deep template-binding cleft formed by the palm, fingers, and thumb subdomains. The polymerase domain is linked to RNaseH by the connection subdomain. The active site, located in the palm, contains three critical Asp residues (110, 185, and 186) in close proximity, and two coordinated Mg^{2+} ions. Mutation of these Asp residues abolishes RT polymerizing activity. The orientation of the three active-site Asp residues is similar to that observed in other DNA polymerases (e.g., the Klenow fragment of *E. coli* DNA polI). The p51 subunit appears to be rigid and does not form a polymerizing cleft; Asp 110, 185, and 186 of this subunit are buried within the molecule. Approximately 18 base pairs of the primer-template duplex lie in the nucleic acid binding cleft, stretching from the polymerase active site to the RNaseH domain.

In the RT-primer-template-dNTP structure, the presence of a dideoxynucleotide at the 3' end of the primer allows visualization of the catalytic complex trapped just prior to attack on the incoming dNTP. Comparison with previously obtained structures suggests a model whereby the fingers close in to trap the template and dNTP prior to nucleophilic attack of the 3'-OH of the primer on the incoming dNTP. After the addition of the incoming dNTP to the growing chain, it has been proposed that the fingers adopt a more open configuration, thereby releasing the pyrophosphate and enabling RT to bind the next dNTP. The structure of the HIV-1 RNaseH has also been determined by x-ray crystallography; this domain displays a global folding similar to that of *E. coli* RNaseH.

Integrase

A distinguishing feature of retrovirus replication is the insertion of a DNA copy of the viral genome into the host cell chromosome following reverse transcription. The integrated viral DNA (the provirus) serves as the template for the synthesis of viral RNAs and is maintained as part of the host cell genome for the lifetime of the infected cell. Retroviral mutants deficient in the ability to integrate generally fail to establish a productive infection.

The integration of viral DNA is catalyzed by integrase, a 32-kd protein generated by PR-mediated cleavage of the C-terminal portion of the HIV-1 Gag-Pol polyprotein (see Fig. IV).

Retroviral IN proteins are composed of three structurally and functionally distinct domains: an N-terminal, zinc-finger-containing domain, a core domain, and a relatively nonconserved C-terminal domain. Because of its low solubility, it has not yet been possible to crystallize the entire 288-amino-acid HIV-1 IN protein. However, the structure of all three domains has been solved independently by x-ray crystallography or NMR methods. The crystal structure of the core domain of the avian sarcoma virus IN has also been determined. The N-terminal domain (residues 1 to 55), whose structure was solved by NMR spectroscopy, is composed of four helices with a zinc coordinated by amino acids His-12, His-16, Cys-40, and Cys-43. The structure of the N-terminal domain is reminiscent of helical DNA binding proteins that contain a so-called helix-turn-helix motif; however, in the HIV-1 structure this motif contributes to dimer formation. Initially, poor solubility hampered efforts to solve the structure of the core domain. However, attempts at crystallography were successful when it was observed that a Phe-to-Lys change at IN residue 185 greatly increased solubility without disrupting *in vitro* catalytic activity. Each monomer of the HIV-1 IN core domain (IN residues 50 to 212) is composed of a five-stranded β-sheet flanked by helices; this structure bears striking resemblance to other polynucleotidyl transferases including RNaseH and the bacteriophage MuA transposase. Three highly conserved residues are found in analogous positions in other polynucleotidyl transferases; in HIV-1 IN these are Asp-64, Asp-116 and Glu-152, the so-called D,D-35-E motif. Mutations at these positions block HIV IN function both *in vivo* and *in vitro*. The close proximity of these three amino acids in the crystal structure of both avian sarcoma virus and HIV-1 core domains supports the hypothesis that these residues play a central role in catalysis of the polynucleotidyl transfer reaction that is at the heart of the integration process. The C-terminal domain, whose structure has been solved by NMR methods, adopts a five-stranded β-barrel folding topology reminiscent of a Src homology 3 (SH3) domain. Recently, the x-ray structures of SIV and Rous sarcoma virus IN protein fragments encompassing both the core and C-terminal domains have been solved.

Env

The HIV Env glycoproteins play a major role in the virus life cycle. They contain the determinants that interact with the CD4 receptor and coreceptor, and they catalyze the fusion reaction between the lipid bilayer of the viral envelope and the host cell plasma membrane. In addition, the HIV Env glycoproteins contain epitopes that elicit immune responses that are important from both diagnostic and vaccine development perspectives.

The HIV Env glycoprotein is synthesized from the singly spliced 4.3-kb Vpu/Env bicistronic mRNA (see Fig. IV); translation occurs on ribosomes associated with the rough endoplasmic reticulum (ER). The 160-kd polyprotein precursor (gp160) is an integral membrane protein that is anchored to cell membranes by a hydrophobic stop-transfer signal in the domain destined to be the mature TM Env glycoprotein, gp41 (Fig. VI). The gp160 is cotranslationally glycosylated, forms disulfide bonds, and undergoes oligomerization in the ER. The predominant oligomeric form appears to be a trimer, although dimers and tetramers are also observed. The gp160 is transported to the Golgi, where, like other retroviral envelope precursor proteins, it is proteolytically cleaved by cellular enzymes to the mature SU glycoprotein gp120 and TM glycoprotein gp41 (see Fig. VI). The cellular enzyme responsible for cleavage of retroviral Env precursors following a highly conserved Lys/Arg-X-Lys/Arg-Arg motif is furin or a furin-like protease, although other enzymes may also catalyze gp160 processing. Cleavage of gp160 is required for Env-induced fusion activity and virus infectivity. Subsequent to gp160 cleavage, gp120 and gp41 form a noncovalent association that is critical for transport of the Env complex from the Golgi to the cell surface. The gp120-gp41 interaction is fairly weak, and a substantial amount of gp120 is shed from the surface of Env-expressing cells.

The HIV Env glycoprotein complex, in particular the SU (gp120) domain, is very heavily glycosylated; approximately half the molecular mass of gp160 is composed of oligosaccharide side chains. During transport of Env from its site of synthesis in the ER to the plasma membrane, many of the side chains are modified by the addition of complex sugars. The numerous oligosaccharide side chains form what could be imagined as a sugar cloud obscuring much of gp120 from host immune recognition. As shown in Figure VI, gp120 contains interspersed conserved (C_1 to C_5) and variable (V_1 to V_5) domains. The Cys residues present in the gp120s of different isolates are highly conserved and form disulfide bonds that link the first four variable regions in large loops.

A primary function of viral Env glycoproteins is to promote a membrane fusion reaction between the lipid bilayers of the viral envelope and host cell membranes. This membrane fusion event enables the viral core to gain entry into the host cell cytoplasm. A number of regions in both gp120 and gp41 have been implicated, directly or indirectly, in Env-mediated membrane fusion. Studies of the HA_2 hemagglutinin protein of the orthomyxoviruses and the F protein of the paramyxoviruses indicated that a highly hydrophobic domain at the N-terminus of these proteins, referred to as the fusion peptide, plays a critical role in membrane fusion. Mutational analyses demonstrated that an analogous domain was located at the N-terminus of the HIV-1, HIV-2, and SIV TM glycoproteins (see Fig. VI). Nonhydrophobic substitutions within this region of gp41 greatly reduced or blocked syncytium formation and resulted in the production of noninfectious progeny virions.

C-terminal to the gp41 fusion peptide are two amphipathic helical domains (see Fig. VI) which play a central role in membrane fusion. Mutations in the N-terminal helix (referred to as the N-helix), which contains a Leu zipper-like heptad repeat motif, impair infectivity and membrane fusion activity, and peptides derived from these sequences exhibit potent antiviral activity in culture. The structure of the ectodomain of HIV-1 and SIV gp41, the two helical motifs in particular, has been the focus of structural analyses in recent years. Structures were determined by x-ray crystallography or NMR spectroscopy either for fusion proteins containing the helical domains, a mixture of peptides derived from the N- and C-helices, or in the case of the SIV structure, the intact gp41 ectodomain sequence from residue 27 to 149. These studies obtained fundamentally similar trimeric structures, in which the two helical domains pack in an antiparallel fashion to generate a six-helix bundle. The N-helices form a coiled-coil in the center of the bundle, with the C-helices packing into hydrophobic grooves on the outside.

In the steps leading to membrane fusion CD4 binding induces conformation changes in Env that facilitate coreceptor binding. Following the formation of a ternary gp120/CD4/coreceptor complex, gp41 adopts a hypothetical conformation that allows the fusion peptide to insert into the target lipid bilayer. The formation of the gp41 six-helix bundle (which involves antiparallel interactions between the gp41 N- and C-helices) brings the viral and cellular membranes together and membrane fusion takes place.

Use of Recombinant MVA Virus To Boost CD+8 Cell Immune Response

The present invention relates to generation of a $CD8^+$ T cell immune response against an antigen and also eliciting an antibody response. More particularly, the present invention relates to "prime and boost" immunization regimes in which the immune response induced by administration of a priming composition is boosted by administration of a boosting composition. The present invention is based on inventors' experimental demonstration that effective boosting can be achieved using modified vaccinia Ankara (MVA) vectors, following priming with any of a variety of different types of priming compositions including recombinant MVA itself.

A major protective component of the immune response against a number of pathogens is mediated by T lymphocytes of the $CD8^+$ type, also known as cytotoxic T lymphocytes (CTL). An important function of $CD8^+$ cells is secretion of gamma interferon (IFNγ), and this provides a measure of $CD8^+$ T cell immune response. A second component of the immune response is antibody directed to the proteins of the pathogen.

The present invention employs MVA which, as the experiments described below show, has been found to be an effective means for providing a boost to a $CD8^+$ T cell immune response primed to antigen using any of a variety of different priming compositions and also eliciting an antibody response.

Remarkably, the experimental work described below demonstrates that use of embodiments of the present invention allows for recombinant MVA virus expressing an HIV antigen to boost a $CD8^+$ T cell immune response primed by a DNA vaccine and also eliciting an antibody response. The MVA was found to induce a $CD8^+$ T cell response after intradermal, intramuscular or mucosal immunization. Recombinant MVA has also been shown to prime an immune response that is boosted by one or more inoculations of recombinant MVA.

Non-human primates immunized with plasmid DNA and boosted with the MVA were effectively protected against intramucosal challenge with live virus. Advantageously, the inventors found that a vaccination regime used intradermal, intramuscular or mucosal immunization for both prime and boost can be employed, constituting a general immunization regime suitable for inducing $CD8^+$ T cells and also eliciting an antibody response, e.g. in humans.

The present invention in various aspects and embodiments employs an MVA vector encoding an HIV antigen for boosting a $CD8^+$ T cell immune response to the antigen primed by previous administration of nucleic acid encoding the antigen and also eliciting an antibody response.

A general aspect of the present invention provides for the use of an MVA vecto. for boosting a $CD8^+$ T cell immune response to an HIV antigen and also eliciting an antibody response.

One aspect of the present invention provides a method of boosting a $CD8^+$ T cell immune response to an HIV antigen in an individual, and also eliciting an antibody response, the method including provision in the individual of an MVA vector including nucleic acid encoding the antigen operably linked to regulatory sequences for production of antigen in the individual by expression from the nucleic acid, whereby a $CD8^+$ T cell immune response to the antigen previously primed in the individual is boosted.

An immune response to an HIV antigen may be primed by immunization with plasmid DNA or by infection with an infectious agent.

A further aspect of the invention provides a method of inducing a $CD8^+$ T cell immune response to an HIV antigen in an individual, and also eliciting an antibody response, the method comprising administering to the individual a priming composition comprising nucleic acid encoding the antigen and then administering a boosting composition which comprises an MVA vector including nucleic acid encoding the antigen operably linked to regulatory sequences for production of antigen in the individual by expression from the nucleic acid.

A further aspect provides for use of an MVA vector, as disclosed, in the manufacture of a medicament for administration to a mammal to boost a $CD8^+$ T cell immune response to an HIV antigen, and also eliciting an antibody response. Such a medicament is generally for administration following prior administration of a priming composition comprising nucleic acid encoding the antigen.

The priming composition may comprise any viral vector, such as a vaccinia virus vector such as a replication-deficient strain such as modified vaccinia Ankara (MVA) or NYVAC (Tartaglia *et al.* 1992 *Virology* **118**:217-232), an avipox vector such as fowlpox or canarypox, e.g. the strain known as ALVAC (Paoletti *et al.* 1994 *Dev Biol Stand* **82**:65-69), or an adenovirus vector or a vesicular stomatitis virus vector or an alphavirus vector.

The priming composition may comprise DNA encoding the antigen, such DNA preferably being in the form of a circular plasmid that is not capable of replicating in mammalian cells. Any selectable marker should not be resistance to an antibiotic used clinically, so for example Kanamycin resistance is preferred to Ampicillin resistance. Antigen expression should be driven by a promoter which is active in mammalian cells, for instance the cytomegalovirus immediate early (CMV IE) promoter.

In particular embodiments of the various aspects of the present invention, administration of a priming composition is followed by boosting with a boosting composition, or first and second boosting compositions, the first and second boosting compositions being the same or different from one another. Still further boosting compositions may be employed without departing from the present invention. In one embodiment, a triple immunization regime employs DNA, then adenovirus as a first boosting composition, then MVA as a second boosting composition, optionally followed by a further (third) boosting composition or subsequent boosting administration of one or other or both of the same or different vectors. Another option is DNA then MVA then adenovirus, optionally followed by subsequent boosting administration of one or other or both of the same or different vectors.

The antigen to be encoded in respective priming and boosting compositions (however many boosting compositions are employed) need not be identical, but should share at least one $CD8^+$ T cell epitope. The antigen may correspond to a complete antigen, or a fragment thereof. Peptide epitopes or artificial strings of epitopes may be employed, more efficiently cutting out unnecessary protein sequence in the antigen and encoding sequence in the vector or vectors. One or more additional epitopes may be included, for instance epitopes which are recognized by T helper cells, especially epitopes recognized in individuals of different HLA types.

An HIV antigen of the invention to be encoded by a recombinant MVA virus includes polypeptides having immunogenic activity elicited by an amino acid sequence of an HIV Env, Gag, Pol, Vif, Vpr, Tat, Rev, Vpu, or Nef amino acid sequence as at least one $CD8^+$ T cell epitope. This amino acid sequence substantially corresponds to at least one 10-900 amino acid fragment and/or consensus sequence of a known HIV Env or Pol; or at least one 10-450 amino acid fragment and/or consensus sequence of a known HIV Gag; or at

least one 10-100 amino acid fragment and/or consensus sequence of a known HIV Vif, Vpr, Tat, Rev, Vpu, or Nef.

Although a full length Env precursor sequence is presented for use in the present invention, Env is optionally deleted of subsequences. For example, regions of the gp120 surface and gp41 transmembrane cleavage products can be deleted.

Although a full length Gag precursor sequence is presented for use in the present invention, Gag is optionally deleted of subsequences. For example, regions of the matrix protein (p17), regions of the capsid protein (p24), regions of the nucleocapsid protein (p7), and regions of p6 (the C-terminal peptide of the Gag polyprotein) can be deleted.

Although a full length Pol precursor sequence is presented for use in the present invention, Pol is optionally deleted of subsequences. For example, regions of the protease protein (p10), regions of the reverse transcriptase protein (p66/p51), and regions of the integrase protein (p32) can be deleted.

Such an HIV Env, Gag, or Pol can have overall identity of at least 50% to a known Env, Gag, or Pol protein amino acid sequence, such as 50-99% identity, or any range or value therein, while eliciting an immunogenic response against at least one strain of an HIV.

Percent identify can be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J Mol Biol* 1970 **48**:443), as revised by Smith and Waterman (*Adv Appl Math* 1981 **2**:482). Briefly, the GAP program defines identity as the number of aligned symbols (i.e., nucleotides or amino acids) which are identical, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unitary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov and Burgess (*Nucl Acids Res* 1986 **14**:6745), as described by Schwartz and Dayhoff (eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, Washington, D.C. 1979, pp. 353-358); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

In a preferred embodiment, an Env of the present invention is a variant form of at least one HIV envelope protein. Preferably, the Env is composed of gp120 and the

membrane-spanning and ectodomain of gp41 but lacks part or all of the cytoplasmic domain of gp41.

Known HIV sequences are readily available from commercial and institutional HIV sequence databases, such as GENBANK, or as published compilations, such as Myers *et al.* eds., *Human* Retroviruses *and AIDS, A Compilatio*: *and Analysis of Nucleic Acid and Amino Acid Sequences*, Vol. I and II, Theoretical Biology and Biophysics, Los Alamos, N. Mex. (1993), or http://hiv-web.lanl.gov/.

Substitutions or insertions of an HIV Env, Gag, or Pol to obtain an additional HIV Env, Gag, or Pol, encoded by a nucleic acid for use in a recombinant MVA virus of the present invention, can include substitutions or insertions of at least one amino acid residue (e.g., 1-25 amino acids). Alternatively, at least one amino acid (e.g., 1-25 amino acids) can be deleted from an HIV Env, Gag, or Pol sequence. Preferably, such substitutions, insertions or deletions are identified based on safety features, expression levels, immunogenicity and compatibility with high replication rates of MVA.

Amino acid sequence variations in an HIV Env, Gag, or Pol of the present invention can be prepared e.g., by mutations in the DNA. Such HIV Env, Gag, or Pol include, for example, deletions, insertions or substitutions of nucleotides coding for different amino acid residues within the amino acid sequence. Obviously, mutations that will be made in nucleic acid encoding an HIV Env, Gag, or Pol must not place the sequence out of reading frame and preferably will not create complementary domains that could produce secondary mRNA structures.

HIV Env, Gag, or Pol-encoding nucleic acid of the present invention can also be prepared by amplification or site-directed mutagenesis of nucleotides in DNA or RNA encoding an HIV Env, Gag, or Pol and thereafter synthesizing or reverse transcribing the encoding DNA to produce DNA or RNA encoding an HIV Env, Gag, or Pol, based on the teaching and guidance presented herein.

Recombinant MVA viruses expressing HIV Env, Gag, or Pol of the present invention, include a finite set of HIV Env, Gag, or Pol-encoding sequences as substitution nucleotides that can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein. For a detailed description of protein chemistry and structure, see Schulz, G.E. *et al.*, 1978 *Principles of Protein Structure*, Springer-Verlag, New York, N.Y., and Creighton, T.E., 1983 *Proteins:*

Structure and Molecular Properties, W. H. Freeman & Co., San Francisco, CA. For a presentation of nucleotide sequence substitutions, such as codon preferences, see Ausubel *et al.* eds. *Current Protocols in Molecular Biology*, Greene Publishing Assoc., New York, N.Y. 1994 at §§ A.1.1-A.1.24, and Sambrook, J. *et al.* 1989 *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. at Appendices C and D.

Thus, one of ordinary skill in the art, given the teachings and guidance presented herein, will know how to substitute other amino acid residues in other positions of an HIV *env*, *gag*, or *pol* DNA or RNA to obtain alternative HIV Env, Gag, or Pol, including substitutional, deletional or insertional variants.

Within the MVA vector, regulatory sequences for expression of the encoded antigen will include a natural, modified or synthetic poxvirus promoter. By "promoter" is meant a sequence of nucleotides from which transcription may be initiated of DNA operably linked downstream (i.e. in the 3' direction on the sense strand of double-stranded DNA). "Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter. Other regulatory sequences including terminator fragments, polyadenylation sequences, marker genes and other sequences may be included as appropriate, in accordance with the knowledge and practice of the ordinary person skilled in the art: see, for example, Moss, B. (2001). Poxviridae: the viruses and their replication. In Fields Virology, D.M. Knipe, and P.M. Howley, eds. (Philadelphia, Lippincott Williams & Wilkins), pp. 2849-2883. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Current Protocols in Molecular Biology*, 1998 Ausubel *et al.* eds., John Wiley & Sons.

Promoters for use in aspects and embodiments of the present invention must be compatible with poxvirus expression systems and include natural, modified and synthetic sequences.

Either or both of the priming and boosting compositions may include an adjuvant, such as granulocyte macrophage-colony stimulating factor (GM-CSF) or encoding nucleic acid therefor.

Administration of the boosting composition is generally about 1 to 6 months after administration of the priming composition, preferably about 1 to 3 months.

Preferably, administration of priming composition, boosting composition, or both priming and boosting compositions, is intradermal, intramuscular or mucosal immunization.

Administration of MVA vaccines may be achieved by using a needle to inject a suspension of the virus. An alternative is the use of a needleless injection device to administer a virus suspension (using, e.g., Biojector™ needleless injector) or a resuspended freeze-dried powder containing the vaccine, providing for manufacturing individually prepared doses that do not need cold storage. This would be a great advantage for a vaccine that is needed in rural areas of Africa.

MVA is a virus with an excellent safety record in human immunizations. The generation of recombinant viruses can be accomplished simply, and they can be manufactured reproducibly in large quantities. Intradermal, intramuscular or mucosal administration of recombinant MVA virus is therefore highly suitable for prophylactic or therapeutic vaccination of humans against AIDS which can be controlled by a $CD8^+$ T cell response.

The individual may have AIDS such that delivery of the antigen and generation of a $CD8^+$ T cell immune response to the antigen is of benefit or has a therapeutically beneficial effect.

Most likely, administration will have prophylactic aim to generate an immune response against HIV or AIDS before infection or development of symptoms.

Components to be administered in accordance with the present invention may be formulated in pharmaceutical compositions. These compositions may comprise a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal routes.

As noted, administration is preferably intradermal, intramuscular or mucosal.

Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous, subcutaneous, intramuscular or mucosal injection, or injection at the site of affliction, the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilizers, buffers, antioxidants and/or other additives may be included as required.

A slow-release formulation may be employed.

Following production of MVA particles and optional formulation of such particles into compositions, the particles may be administered to an individual, particularly human or other primate. Administration may be to another mammal, e.g. rodent such as mouse, rat or hamster, guinea pig, rabbit, sheep, goat, pig, horse, cow, donkey, dog or cat.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, or in a veterinary context a veterinarian, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in *Remington's Pharmaceutical Sciences*, 16th edition, 1980, Osol, A. (ed.).

In one preferred regimen, DNA is administered at a dose of 250 μg to 2.5 mg/injection, followed by MVA at a dose of 10^6 to 10^9 infectious virus particles/injection.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Delivery to a non-human mammal need not be for a therapeutic purpose, but may be for use in an experimental context, for instance in investigation of mechanisms of immune responses to an antigen of interest, e.g. protection against HIV or AIDS.

Further aspects and embodiments of the present invention will be apparent to those of ordinary skill in the art, in view of the above disclosure and following experimental

exemplification, included by way of illustration and not limitation, and with reference to the attached figures.

EXAMPLE 1

Control of a Mucosal Challenge and Prevention of AIDS by a Multiprotein DNA/MVA Vaccine

Here we tested DNA priming and poxvirus boosting for the ability to protect against a highly pathogenic mucosal challenge. The 89.6 chimera of simian and human immunodeficiency viruses (SHIV-89.6) was used for the construction of immunogens and its highly pathogenic derivative, SHIV-89.6P, for challenge (G.B. Karlsson *et al.* 1997 *J Virol* **71**:4218). SHIV-89.6 and SHIV-89.6P do not generate cross-neutralizing antibody (D.C... Montefiori *et al.* 1998 *J Virol* **72**:3427) and allowed us to address the ability of vaccine-raised T cells and non-neutralizing antibodies to control an immunodeficiency virus challenge. Modified vaccinia Ankara (MVA) was used for the construction of the recombinant poxvirus. MVA has been highly effective at boosting DNA-primed CD8 T cells and enjoys the safety feature of not replicating efficiently in human or monkey cells (H.L. Robinson *et al.* 2000 *AIDS Reviews* **2**:105).

To ensure a broad immune response both the DNA and recombinant MVA (rMVA) components of the vaccine expressed multiple immunodeficiency virus proteins. The DNA prime (DNA/89.6) expressed simian immunodeficiency virus (SIV) Gag, Pol, Vif, Vpx, and Vpr and human immunodeficiency virus-1 (HIV-1) Env, Tat, and Rev from a single transcript (R.J. Gorelick *et al.* 1999 *Virology* **253**:259; M.M. Sauter *et al.* 1996 *J Cell Biol* **132**:795).

Molecularly cloned SHIV-89.6 sequences were cloned into the vector pGA2 using ClaI and RsrII sites. This cloning deleted both long terminal repeats (LTRs) and *nef*. The SHIV-89.6 sequences also were internally mutated for a 12-base pair region encoding the first four amino acids of the second zinc finger in nucleocapsid. This mutation renders SHIV viruses noninfectious (R.J. Gorelick *et al.* 1999 *Virology* **253**:259). A mutation in gp41 converted the tyrosine at position 710 to cysteine to achieve better expression of Env on the plasma membrane of DNA-expressing cells (M.M. Sauter *et al.* 1996 *J Cell Biol* **132**:795). pGA2 uses the CMV immediate early promoter without intron A and the bovine growth hormone polyadenylation sequence to express vaccine inserts. Vaccine DNA was

produced by Althea (San Diego, CA). In transient transfections of 293T cells, DNA/89.6 produced about 300 ng of Gag and 85 ng of Env per $1x10^6$ cells.

The rMVA booster (MVA/89.6) expressed SIV Gag, Pol, and HIV-1 Env under the control of vaccinia virus early/late promoters.

The MVA double recombinant virus expressed both the HIV 89.6 Env and the SIV 239 Gag-Pol, which were inserted into deletion II and deletion III of MVA, respectively. The 89.6 Env protein was truncated for the COOH-terminal 115 amino acids of gp41. The modified H5 promoter controlled the expression of both foreign genes.

Vaccination was accomplished by priming with DNA at 0 and 8 weeks and boosting with rMVA at 24 weeks (Fig. 1A).

I.d. and i.m. DNA immunizations were delivered in phosphate-buffered saline (PBS) with a needleless jet injector (Bioject, Portland, OR) to deliver five i.d. 100-µl injections to each outer thigh for the 2.5-mg dose of DNA or one i.d. 100-µl injection to the right outer thigh for the 250-µg dose of plasmid. I.m. deliveries of DNA were done with one 0.5-ml injection of DNA in PBS to each outer thigh for the 2.5-mg dose and one 100-µl injection to the right outer thigh for the 250-µg dose. $1x10^8$ pfu of MVA/89.6 was administered both i.d. and i.m. with a needle. One 100-µl dose was delivered to each outer thigh for the i.d. dose and one 500-µl dose to each outer thigh for the i.m dose. Control animals received 2.5 mg of the pGA2 vector without vaccine insert with the Bioject device to deliver five 100-µl doses i.d. to each outer thigh. The control MVA booster immunization consisted of $2x10^8$ pfu of MVA without an insert delivered i.d. and i.m. as described for MVA/89.6.

Four groups of six rhesus macaques each were primed with either 2.5 mg (high-dose) or 250 µg (low-dose) of DNA by intradermal (i.d.) or intramuscular (i.m.) routes using a needleless jet injection device (Bioject, Portland, OR) (T.M. Allen *et al.* 2000 *J Immunol* **164**:4968).

Young adult rhesus macaques from the Yerkes breeding colony were cared for under guidelines established by the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals" with protocols approved by the Emory University Institutional Animal Care and Use Committee. Macaques were typed for the *Mamu-A*01* allele with polymerase chain reaction (PCR) analyses (M.A. Egan *et al.* 2000 *J Virol* **74**:7485; I. Ourmanov *et al.* 2000 *J Virol* **74**:2740). Two or more animals containing at

least one *Mamu-A*01* allele were assigned to each group. Animal numbers are as follows: 1, RBr-5*; 2, RIm-5*; 3, RQf-5*; 4, RZe-5; 5, ROm-5; 6, RDm-5; 7, RAj-5*; 8, RJi-5*; 9, RAl-5*; 10, RDe-5*; 11, RAi-5; 12, RPr-5; 13, RKw-4*; 14, RWz-5*; 15, RGo-5; 16, RLp-4; 17, RWd-6; 18, RAt-5; 19, RPb-5*; 20, RIi-5*; 21, RIq-5; 22, RSp-4; 23, RSn-5; 24, RGd-6; 25, RMb-5*; 26, RGy-5*; 27, RUs-4; and 28, RPm-5. Animals with the *A*01* allele are indicated with asterisks.

Gene gun deliveries of DNA were not used because these had primed non-protective immune responses in a 1996 - 98 trial (H.L. Robinson *et al.* 1999 *Nat Med* 5:526). The MVA/89.6 booster immunization ($2x10^8$ plaque-forming units, pfu) was injected with a needle both i.d. and i.m. A control group included two mock immunized animals and two naive animals. The challenge was given at 7 months after the rMVA booster to test for the generation of long-term immunity. Because most HIV-1 infections are transmitted across mucosal surfaces, an intrarectal challenge was administered.

DNA priming followed by rMVA boosting generated high frequencies of virus-specific T cells that peaked at one week following the rMVA booster (Fig. 1). The frequencies of T cells recognizing the Gag-CM9 epitope were assessed by means of Mamu-A*01 tetramers, and the frequencies of T cells recognizing epitopes throughout Gag were assessed with pools of overlapping peptides and an enzyme-linked immunospot (ELISPOT) assay (C.A. Power *et al.* 1999 *J Immunol Methods* **227**:99).

For tetramer analyses, about $1x10^6$ peripheral blood mononuclear cells (PBMC) were surface-stained with antibodies to CD3 conjugated to fluorescein isothiocyanate (FITC) (FN-18; Biosource International, Camarillo, CA), CD8 conjugated to peridinin chlorophyl protein (PerCP) (SK1; Becton Dickinson, San Jose, CA), and Gag-CM9 (CTPYDINQM)-*Mamu-A*01* tetramer (SEQ ID NO: 6) conjugated to allophycocyanin (APC), in a volume of 100 µl at 8° to 10°C for 30 min. Cells were washed twice with cold PBS containing 2% fetal bovine serum (FBS), fixed with 1% paraformaldehyde in PBS, and analyzed within 24 hrs on a FACScaliber (Becton Dickinson, San Jose, CA). Cells were initially gated on lymphocyte populations with forward scatter and side scatter and then on CD3 cells. The CD3 cells were then analyzed for CD8 and tetramer-binding cells. About 150,000 lymphocytes were acquired for each sample. Data were analyzed using FloJo software (Tree Star, San Carlos, CA).

For interferon-γ (IFN-γ) ELISPOTs, MULTISCREEN 96 well filtration plates (Millipore Inc. Bedford, MA) were coated overnight with antibody to human IFN-γ (Clone B27, Pharmingen, San Diego, CA) at a concentration of 2 μg/ml in sodium bicarbonate buffer (pH 9.6) at 8° to 10°C. Plates were washed two times with RPMI medium and then blocked for 1 hour with complete medium (RPMI containing 10% FBS) at 37°C. Plates were washed five more times with plain RPMI medium, and cells were seeded in duplicate in 100 μl complete medium at numbers ranging from $2x10^4$ to $5x10^5$ cells per well. Peptide pools were added to each well to a final concentration of 2 μg/ml of each peptide in a volume of 100 μl in complete medium. Cells were cultured at 37°C for about 36 hrs under 5% CO_2. Plates were washed six times with wash buffer (PBS with 0.05% Tween-20) and then incubated with 1 μg of biotinylated antibody to human IFN-γ per milliliter (clone 7-86-1; Diapharma Group, West Chester, OH) diluted in wash buffer containing 2% FBS. Plates were incubated for 2 hrs at 37°C and washed six times with wash buffer. Avidin-horseradish peroxidase (Vector Laboratories, Burlingame, CA) was added to each well and incubated for 30 to 60 min at 37°C. Plates were washed six times with wash buffer and spots were developed using stable DAB as substrate (Research Genetics, Huntsville, AL). Spots were counted with a stereo dissecting microscope. An ovalbumin peptide (SIINFEKL) (SEQ ID NO: 7) was included as a control in each analysis. Background spots for the ovalbumin peptide were generally <5 for $5x10^5$ PBMCs. This background when normalized for $1x10^6$ PBMC was <10. Only ELISPOT counts of twice the background (≥20) were considered significant. The frequencies of ELISPOTs are approximate because different dilutions of cells have different efficiencies of spot formation in the absence of feeder cells (C.A. Power *et al.* 1999 *J Immunol Methods* **227**: 99). The same dilution of cells was used for all animals at a given time point, but different dilutions were used to detect memory and acute responses.

Gag-CM9 tetramer analyses were restricted to macaques that expressed the *Mamu-A*01* histocompatibility type, whereas ELISPOT responses did not depend on a specific histocompatibility type. As expected, the DNA immunizations raised low levels of memory cells that expanded to high frequencies within 1 week of the rMVA booster (Fig. 1 and 6). In *Mamu-A*01* macaques, CD8 cells specific to the Gag-CM9 epitope expanded to frequencies as high as 19% of total CD8 T cells (Fig. 6). This peak of specific cells underwent a 10- to 100-fold contraction into the DNA/MVA memory pool (Fig. 1A and 6).

ELISPOTs for three pools of Gag peptides also underwent a major expansion (frequencies up to 4000 spots for $1x10^6$ PBMC) before contracting from 5- to 20-fold into the DNA/MVA memory response (Fig. 1B). The frequencies of ELISPOTs were the same in macaques with and without the *A*01* histocompatibility type (P>0.2).

Simple linear regression was used to estimate correlations between postbooster and postchallenge ELISPOT responses, between memory and postchallenge ELISPOT responses, and between logarithmically transformed viral loads and ELISPOT frequencies. Comparisons between vaccine and control groups and *A*01* and non *A*01* macaques were performed by means of two-sample *t* tests with logarithmically transformed viral load and ELISPOT responses. Two-way analyses of variance were used to examine the effects of dose and route of administration on peak DNA/MVA ELISPOTs, on memory DNA/MVA ELISPOTs, and on logarithmically transformed Gag antibody data.

At both peak and memory phases of the vaccine response, the rank order for the height of the ELISPOTs in the vaccine groups was 2.5 mg i.d. > 2.5 mg i.m. > 250 μg i.d. > 250 μg i.m. (Fig. 1B). The IFN-γ ELISPOTs included both CD4 and CD8 cells. Gag-CM9-specific CD8 cells had good lytic activity after restimulation with peptide.

The highly pathogenic SHIV-89.6P challenge was administered intrarectally at 7 months after the rMVA booster, when vaccine-raised T cells were in memory (Fig. 1).

The challenge stock (5.7×10^9 copies of viral RNA per milliliter) was produced by one intravenous followed by one intrarectal passage in rhesus macaques of the original SHIV-89.6P stock (G.B. Karlsson *et al.* 1997 *J Virol* **71**:4218). Lymphoid cells were harvested from the intrarectally infected animal at peak viremia, CD8-depleted, and mitogen-stimulated for stock production. Before intrarectal challenge, fasted animals were anesthetized (ketamine, 10 mg/kg) and placed on their stomach with the pelvic region slightly elevated. A feeding tube (8Fr (2.7 mm) x 16 inches (41 cm); Sherwood Medical, St. Louis, MO) was inserted into the rectum for a distance of 15 to 20 cm. Following insertion of the feeding tube, a syringe containing 20 intrarectal infectious doses in 2 ml of RPMI-1640 plus 10% FBS was attached to the tube and the inoculum was slowly injected into the rectum. After delivery of the inoculum, the feeding tube was flushed with 3.0 ml of RPMI without FBS and then slowly withdrawn. Animals were left in place, with pelvic regions slightly elevated, for a period of ten minutes after the challenge.

The challenge infected all of the vaccinated and control animals (Fig. 2). However, by 2 weeks after challenge, titers of plasma viral RNA were at least 10-fold lower in the vaccine groups (geometric means of $1x10^7$ to $5x10^7$) than in the control animals (geometric mean of $4x10^8$) (Fig. 2A) (S. Staprans *et al.* in: *Viral Genome Methods* K. Adolph, ed. CRC Press, Boca Raton, FL, 1996 pp. 167-184; R. Hofmann-Lehmann *et al.* 2000 *AIDS Res Hum Retroviruses* **16**:1247).

For the determination of SHIV copy number, viral RNA from 150 µl of ACD anticoagulated plasma was directly extracted with the QIAamp Viral RNA kit (Qiagen), eluted in 60 µl of AVE buffer, and frozen at -80°C until SHIV RNA quantitation was performed. Five microliters of purified plasma RNA was reverse-transcribed in a final 20-µl volume containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate (dNTP), 2.5 µM random hexamers, 20 units MultiScribe RT, and 8 units ribonuclease inhibitor. Reactions were incubated at 25°C for 10 min, followed by incubation at 42°C for 20 min, and inactivation of reverse transcriptase at 99°C for 5 min. The reaction mix was adjusted to a final volume of 50 µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4 mM $MgCl_2$, 0.4 mM each dNTP, 0.2 µM forward primer, 0.2 µM reverse primer, 0.1 µM probe, and 5 units AmpliTaq Gold DNA polymerase (all reagents from PerkinElmer Applied Biosystems, Foster City, CA). The primer sequences within a conserved portion of the SIV *gag* gene are the same as those described previously (S. Staprans *et al.* in: *Viral Genome Methods* K. Adolph, ed. CRC Press, Boca Raton, FL, 1996 pp. 167-184). A PerkinElmer Applied Biosystems 7700 Sequence Detection System was used with the PCR profile: 95°C for 10 min, followed by 40 cycles at 93°C for 30 s, and 59.5°C for 1 min. PCR product accumulation was monitored with the 7700 sequence detector and a probe to an internal conserved *gag* gene sequence: 6FAM-CTGTCTGCGTCATTTGGTGC-Tamra (SEQ ID NO: 8), where FAM and Tamra denote the reporter and quencher dyes. SHIV RNA copy number was determined by comparison with an external standard curve consisting of virion-derived SIVmac239 RNA quantified by the SIV bDNA method (Bayer Diagnostics, Emeryville, CA). All specimens were extracted and amplified in duplicate, with the mean result reported. With a 0.15-ml plasma input, the assay has a sensitivity of 10^3 RNA copies per milliliter of plasma and a linear dynamic range of 10^3 to 10^8 RNA copies ($R^2 = 0.995$). The intraassay coefficient of variation was <20% for samples containing $>10^4$ SHIV RNA copies per milliliter, and

<25% for samples containing 10^3 to 10^4 SHIV RNA copies per milliliter. To more accurately quantitate low SHIV RNA copy number in vaccinated animals at weeks 16 and 20, we made the following modifications to increase the sensitivity of the SHIV RNA assay: (i) Virions from ≤1 ml of plasma were concentrated by centrifugation at 23,000g at 10°C for 150 min before viral RNA extraction, and (ii) a one-step reverse transcriptase PCR method was used (R. Hofmann-Lehmann *et al.* 2000 *AIDS Res Hum Retroviruses* **16**:1247). These changes provided a reliable quantification limit of 300 SHIV RNA copies per milliliter, and gave SHIV RNA values that were highly correlated to those obtained by the first method used ($r = 0.91$, $P<0.0001$).

By 8 weeks after challenge, both high-dose DNA-primed groups and the low-dose i.d. DNA-primed group had reduced their geometric mean loads to about 1000 copies of viral RNA per milliliter. At this time, the low-dose i.m. DNA-primed group had a geometric mean of $6x10^3$ copies of viral RNA and the nonvaccinated controls had a geometric mean of 2×10^6. By 20 weeks after challenge, even the low-dose i.m. group had reduced its geometric mean copies of viral RNA to 1000. Among the 24 vaccinated animals, only one animal, animal number 22 in the low-dose i.m. group, had intermittent viral loads above $1x10^4$ copies per milliliter (Fig 2D).

By 5 weeks after challenge, all of the nonvaccinated controls had undergone a profound depletion of CD4 cells (Fig 2B). All of the vaccinated animals maintained their CD4 cells, with the exception of animal 22 in the low dose i.m. group (see above), which underwent a slow CD4 decline (Fig. 2E). By 23 weeks after challenge, three of the four control animals had succumbed to AIDS (Fig. 2C). These animals had variable degrees of enterocolitis with diarrhea, cryptosporidiosis, colicystitis, enteric campylobacter infection, splenomegaly, lymphadenopathy, and SIV-associated giant cell pneumonia. In contrast, all 24 vaccinated animals maintained their health.

Containment of the viral challenge was associated with a burst of antiviral T cells (Fig. 1 and 3A). At one week after challenge, the frequency of tetramer$^+$ cells in the peripheral blood had decreased, potentially reflecting the recruitment of specific T cells to the site of infection (Fig. 3A). However, by two weeks after challenge, tetramer$^+$ cells in the peripheral blood had expanded to frequencies as high as, or higher than, after the rMVA booster (Fig. 1 and 3A). The majority of the tetramer$^+$ cells produced IFN-γ in response to a 6-hour peptide stimulation (Fig. 3B) (S.L. Waldrop *et al.* 1997 *J Clin Invest* **99**:1739) and

did not have the "stunned" IFN-γ negative phenotype sometimes observed in viral infections (F. Lechner *et al.* 2000 *J Exp Med* **191**:1499).

For intracellular cytokine assays, about $1x10^6$ PBMC were stimulated for 1 hour at 37°C in 5 ml polypropylene tubes with 100 μg of Gag-CM9 peptide (CTPYDINQM) (SEQ ID NO: 6) per milliliter in a volume of 100 μl RPMI containing 0.1% bovine serum albumin (BSA) and 1 μg of antibody to human CD28 and 1 μg of antibody to human CD49d (Pharmingen, San Diego, CA) per milliliter. Then, 900 μl of RPMI containing 10% FBS and monensin (10 μg/ml) was added, and the cells were cultured for an additional 5 hrs at 37°C at an angle of 5° under 5% CO_2. Cells were surface stained with antibodies to CD8 conjugated to PerCP (clone SK1, Becton Dickinson) at 8° to 10°C for 30 min, washed twice with cold PBS containing 2% FBS, and fixed and permeabilized with Cytofix/Cytoperm solution (Pharmingen). Cells were then incubated with antibodies to human CD3 (clone FN-18; Biosource International, Camarillo, CA) and IFN-γ (Clone B27; Pharmingen) conjugated to FITC and phycoerythrin, respectively, in Perm wash solution (Pharmingen) for 30 min at 4°C. Cells were washed twice with Perm wash, once with plain PBS, and resuspended in 1% paraformaldehyde in PBS. About 150,000 lymphocytes were acquired on the FACScaliber and analyzed with FloJo software.

The postchallenge burst of T cells contracted concomitant with the decline of the viral load. By 12 weeks after challenge, virus-specific T cells were present at about one-tenth of their peak height (Figs. 1A and 3A). In contrast to the vigorous secondary response in the vaccinated animals, the naive animals mounted a modest primary response (Fig. 1B and 3A). Tetramer$^+$ cells peaked at less than 1% of total CD8 cells (Fig. 3A), and IFN-γ-producing ELISPOTs were present at a mean frequency of about 300 as opposed to the much higher frequencies of 1000 to 6000 in the vaccine groups (Fig. 1B) ($P<0.05$).

The tetramer$^+$ cells in the control group, like those in the vaccine group, produced IFN-γ after peptide stimulation (Fig. 3B). By 12 weeks after challenge, three of the four controls had undetectable levels of IFN-γ-producing ELISPOTs. This rapid loss of antiviral T cells in the presence of high viral loads may reflect the lack of CD4 help.

T cell proliferative responses demonstrated that virus-specific CD4 cells had survived the challenge and were available to support the antiviral immune response (Fig. 3C).

About 0.2 million PBMC were stimulated in triplicate for 5 days with the indicated antigen in 200 µl of RPMI at 37°C under 5% CO_2. Supernatants from 293T cells transfected with DNA expressing either SHIV-89.6 Gag and Pol or SHIV-89.6 Gag, Pol and Env were used directly as antigens (final concentration of ~0.5 µg of p27 Gag per milliliter). Supernatants from mock DNA (vector alone)-transfected cells served as negative controls. On day six, cells were pulsed with 1 µCi of tritiated thymidine per well for 16 to 20 hours. Cells were harvested with an automated cell harvester (TOMTEC, Harvester 96, Model 1010, Hamden, CT) and counted with a Wallac 1450 MICROBETA Scintillation counter (Gaithersburg, MD). Stimulation indices are the counts of tritiated thymidine incorporated in PBMC stimulated with 89.6 antigens divided by the counts of tritiated thymidine incorporated by the same PBMC stimulated with mock antigen.

At 12 weeks after challenge, mean stimulation indices for Gag-Pol-Env or Gag-Pol proteins ranged from 35 to 14 in the vaccine groups but were undetectable in the control group. Consistent with the proliferation assays, intracellular cytokine assays demonstrated the presence of virus-specific CD4 cells in vaccinated but not control animals. The overall rank order of the vaccine groups for the magnitude of the proliferative response was 2.5 mg i.d. > 2.5 mg i.m. > 250 µg i.d. > 250 µg i.m.

At 12 weeks after challenge, lymph nodes from the vaccinated animals were morphologically intact and responding to the infection, whereas those from the infected controls had been functionally destroyed (Fig. 4). Nodes from vaccinated animals contained large numbers of reactive secondary follicles with expanded germinal centers and discrete dark and light zones (Fig. 4A). By contrast, lymph nodes from the non-vaccinated control animals showed follicular and paracortical depletion (Fig. 4B), while those from unvaccinated and unchallenged animals displayed normal numbers of minimally reactive germinal centers (Fig. 4C). Germinal centers occupied < 0.05% of total lymph node area in the infected controls, 2% of the lymph node area in the uninfected controls, and up to 18% of the lymph node area in the vaccinated groups (Fig. 4D). More vigorous immune reactivity in the low-dose than the high-dose DNA-primed animals was suggested by more extensive germinal centers in the low dose group (Fig. 4D). At 12 weeks after challenge, *in situ* hybridization for viral RNA revealed rare virus-expressing cells in lymph nodes from 3 of the 24 vaccinated macaques, whereas virus-expressing cells were readily detected in lymph nodes from each of the infected control animals. In the controls, which had

undergone a profound depletion in CD4 T cells, the cytomorphology of infected lymph node cells was consistent with a macrophage phenotype.

The prime/boost strategy raised low levels of antibody to Gag and undetectable levels of antibody to Env (Fig. 5). Postchallenge, antibodies to both Env and Gag underwent anamnestic responses with total Gag antibody reaching heights approaching 1 mg/ml and total Env antibody reaching heights of up to 100 μg/ml.

Enzyme-linked immunosorbent assays (ELISAs) for total antibody to Gag used bacterially produced SIV gag p27 to coat wells (2 μg per milliliter in bicarbonate buffer). ELISAs for antibody to Env antibody used 89.6 Env produced in transiently transfected 293T cells and captured with sheep antibody against Env (catalog number 6205; International Enzymes, Fairbrook CA). Standard curves for Gag and Env ELISAs were produced with serum from a SHIV-89.6-infected macaque with known amounts of immunoglobulin G (IgG) specific for Gag or Env. Bound antibody was detected with peroxidase-conjugated goat antibody to macaque IgG (catalog # YNGMOIGGFCP; Accurate Chemical, Westbury, NY) and TMB substrate (Catalog # T3405; Sigma, St. Louis, MO). Sera were assayed at threefold dilutions in duplicate wells. Dilutions of test sera were performed in whey buffer (4% whey and 0.1% tween 20 in 1X PBS). Blocking buffer consisted of whey buffer plus 0.5% nonfat dry milk. Reactions were stopped with 2M H_2SO_4 and the optical density read at 450 nm. Standard curves were fitted and sample concentrations were interpolated as μg of antibody per ml of serum using SOFTmax 2.3 software (Molecular Devices, Sunnyvale, CA).

By 2 weeks after challenge, neutralizing antibodies for the 89.6 immunogen, but not the SHIV-89.6P challenge, were present in the high-dose DNA-primed groups (geometric mean titers of 352 in the i.d. and 303 in the i.m. groups) (Fig. 5C) (D.C. Montefiori *et al.* 1988 *J Clin* Microbiol **26**:231). By 5 weeks after challenge, neutralizing antibody to 89.6P had been generated (geometric mean titers of 200 in the high-dose i.d. and 126 in the high-dose i.m. group) (Fig. 5D) and neutralizing antibody to 89.6 had started to decline. By 16 to 20 weeks after challenge, antibodies to Gag and Env had fallen in most animals.

Our results demonstrate that a multiprotein DNA/MVA vaccine can raise a memory immune response capable of controlling a highly virulent mucosal immunodeficiency virus challenge. Our levels of viral control were more favorable than have been achieved using only DNA (M.A. Egan *et al.* 2000 *J Virol* 74:7485) or rMVA vaccines (I. Ourmanov *et al.*

2000 *J Virol* **74**:2740) and were comparable to those obtained for DNA immunizations adjuvanted with interleukin-2 (D.H. Barouch *et al.* 2000 *Science* **290**:486). All of these previous studies have used more than three vaccine inoculations, none have used mucosal challenges, and most have challenged at peak effector responses and not allowed a prolonged post vaccination period to test for "long term" efficacy.

The dose of DNA had statistically significant effects on both cellular and humoral responses ($P<0.05$), whereas the route of DNA administration affected only humoral responses. Intradermal DNA delivery was about 10 times more effective than i.m. inoculations for generating antibody to Gag ($P = 0.02$). Neither route nor dose of DNA appeared to have a significant effect on protection. At 20 weeks after challenge, the high-dose DNA-primed animals had slightly lower geometric mean levels of viral RNA ($7x10^2$ and $5x10^2$) than the low-dose DNA-primed animals ($9x10^2$ and $1x10^3$).

The DNA/MVA vaccine controlled the infection, rapidly reducing viral loads to near or below 1000 copies of viral RNA per milliliter of blood. Containment, rather than prevention of infection, affords the opportunity to establish a chronic infection (H.L. Robinson *et al.* 1999 *Nat Med* **5**:526). By rapidly reducing viral loads, a multiprotein DNA/MVA vaccine will extend the prospect for long-term non-progression and limit HIV transmission. (J.W. Mellors *et al.* 1996 *Science* **272**:1167; T.C. Quinn *et al.* 2000 *N Engl J Med* **342**:921).

EXAMPLE 2

MVA Expressing Modified HIV Env, Gag, and Pol Genes

This disclosure describes the construction of a modified vaccinia Ankara (MVA) recombinant virus, MVA/HIV clade B recombinant virus expressing the HIV strain ADA env and the HXB2 gag pol (MVA/HIV ADA env + HXB2 gag pol). For amplification, the lab name of MVA/HIV 48 will be used, which denotes the plasmid from which the construct comes.

The HIV *gag-pol* genes were derived from the Clade B infectious HXB2 virus. The *gag-pol* gene was truncated so that most of the integrase coding sequences were removed and amino acids 185, 266, and 478 were mutated to inactivate reverse transcriptase, inhibit strand transfer activity, and inhibit the RNaseH activity, respectively. The Clade B CCR5 tropic envelope gene was derived from the primary ADA isolate; TTTTTNT sequences were mutated without changing coding capacity to prevent premature transcription

termination and the cytoplasmic tail was truncated in order to improve surface expression, immunogenicity, and stability of the MVA vector. The HIV genes were inserted into a plasmid transfer vector so that *gag-pol* gene was regulated by the modified H5 early/late vaccinia virus promoter and the *env* gene was regulated by the newly designed early/late Psyn II promoter to provide similar high levels of expression. A self-deleting GUS reporter gene was included to allow detection and isolation of the recombinant virus. The HIV genes were flanked by MVA sequences to allow homologous recombination into the deletion 3 site so that the recombinant MVA would remain TK positive for stability and high expression in resting cells. The recombinant MVA was isolated and shown to express abundant amounts of *gag-pol-env* and to process *gag*. Production of HIV-like particles was demonstrated by centrifugation and by electron microscopy. The presence of env in the HIV-like particles was demonstrated by immunoelectron microscopy.

Table of Sequences

Description	SEQ ID NO	FIG. NO
pLW-48	1	A
pLW-48	1	B
Psyn II promoter	2	B
ADA envelope truncated	3	B
PmH5 promoter	4	B
HXB2 *gag pol*	5	B

Plasmid Transfer Vector

The plasmid transfer vector used to make the MVA recombinant virus, pLW-48, (Figure C) by homologous recombination was constructed as follows:

1. From the commercially obtained plasmid, pGem-4Z (Promega), flanking areas on either side of deletion III, designated flank 1 and flank 2, containing 926 and 520 base pairs respectively, were amplified by PCR from the MVA stains of vaccinia virus. Within these flanks, a promoter, the mH5, which had been modified from the originally published sequence by changing two bases that had been shown by previously published work to increase the expression of the cloned gene, was added.

2. A clade B gag pol (Figure D) was truncated so that the integrase was removed and was cloned into the plasmid so that it was controlled by the mH5 promoter. This gene contained the complete HXB2 sequence of the gag. The pol gene has reverse transcriptase safety mutations in amino acid 185 within the active site of RT, in amino acid 266 which inhibits strand transfer activity, and at amino acid 478 which inhibits the RNaseH activity. In addition, the integrase gene was deleted past EcoRI site.

3. A direct repeat of 280 basepairs, corresponding to the last 280 base pairs of MVA flank 1, was added after flank 1.

4. The p11 promoter and GUS reporter gene were added between the two direct repeats of flank 1 so that this screening marker could initially be used for obtaining the recombinant virus, yet deleted out in the final recombinant virus (Scheiflinger, F. *et al.* 1998 *Arch Virol* **143**:467-474; Carroll, M.W. and B. Moss 1995 *BioTechniques* **19**:352-355).

5. A new promoter, Psyn II, was designed to allow for increased expression of the ADA env. The sequence of this new early/late promoter is given in Figure E.

6. A truncated version of the ADA envelope with a silent 5TNT mutation was obtained by PCR and inserted in the plasmid under the control of the Psyn II promoter. The envelope was truncated in the cytoplasmic tail of the gp41 gene, deleting 115 amino acids of the cytoplasmic tail. This truncation was shown to increase the amount of envelope protein on the surface of infected cells and enhance immunogenicity of the envelope protein in mice, and stability of the recombinant virus in tissue culture.

Recombinant MVA Construction

1. MVA virus, which may be obtained from ATCC Number VR-1508, was plaque purified three times by terminal dilutions in chicken embryo fibroblasts (CEF),which were made from 9 day old SPF Premium SPAFAS fertile chicken eggs, distributed by B and E Eggs, Stevens, PA.

2. Secondary CEF cells were infected at an MOI of 0.05 of MVA and transfected with 2 μg of pLW-48, the plasmid described above. Following a two day incubation at 37°C, the virus was harvested, frozen and thawed 3x, and plated out on CEF plates.

3. At 4 days, those foci of infection that stained blue after addition of X-gluc substrate, indicating that recombination had occurred between the plasmid and the infecting

virus, were picked and inoculated on CEF plates. Again, those foci that stained blue were picked.

4. These GUS containing foci were plated out in triplicate and analyzed for GUS staining (which we wanted to now delete) and ADA envelope expression. Individual foci were picked from the 3rd replicate plates of those samples that had about equal numbers of mixed populations of GUS staining and nonstaining foci as well as mostly envelope staining foci.

5. These foci were again plated out in triplicate, and analyzed the same way. After 5 passages, a virus was derived which expressed the envelope protein but which had deleted the GUS gene because of the double repeat. By immunostaining, this virus also expressed the gag pol protein.

Characterization of MVA Recombinant Virus, MVA/HIV 48

1. Aliquots of MVA/HIV 48 infected cell lysates were analyzed by radioimmunoprecipitation and immunostaining with monoclonal antibodies for expression of both the envelope and gag pol protein. In both of these tests, each of these proteins was detected.

2. The recombinant virus was shown to produce gag particles in the supernatant of infected cells by pelleting the ^{35}S-labeled particles on a 20% sucrose cushion.

3. Gag particles were also visualized both outside and budding from cells as well as within vacuoles of cells in the electron microscope in thin sections. These gag particles had envelope protein on their surface.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer, and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the

actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Summary

In summary, we have made a recombinant MVA virus, MVA/HIV 48, which has high expression of the ADA truncated envelope and the HXB2 gag pol. The MVA recombinant virus is made using a transiently expressed GUS marker that is deleted in the final virus. High expression of the ADA envelope is possible because of a new hybrid early/late promoter, Psyn II. In addition, the envelope has been truncated because we have shown truncation of the envelope enhances the amount of protein on the surface of the infected cells, and hence enhances immunogenicity; stability of the recombinant is also enhanced. The MVA recombinant makes gag particles which has been shown by pelleting the particles through sucrose and analyzing by PAGE. Gag particles with envelope protein on the surface have also been visualized in the electron microscope.

EXAMPLE 3

Additional Modified or Synthetic Promoters Designed for Gene Expression in MVA Or Other Poxviruses

Additional modified or synthetic promoters were designed for gene expression in MVA or other poxviruses. Promoters were modified to allow expression at early and late times after infection and to reduce possibility of homologous recombination between identical sequences when multiple promoters are used in same MVA vector. Promoters are placed upstream of protein coding sequence.

m7.5 promoter (SEQ ID NO:10):

CGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAATACAATAATTAATTTCT
CGTAAAAATTGAAAAACTATTCTAATTTATTGCACGGT

Psyn II promoter (SEQ ID NO:2):

TAAAAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATGCTATAAATAATAAATA

Psyn III promoter (SEQ ID NO:11):

TAAAAATTGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATACTATAAATAATAAATA

Psyn IV promoter (SEQ ID NO:12):

TAAAAATTGAAAAACTATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCT
ATACTATAAATAATAAATA

PsynV promoter (SEQ ID NO:13):

AAAAAATGATAAAGTAGGTTCAGTTTTATTGCTGGTTTAAAATCACGCTTTCGA
GTAAAAACTACGAATATAAAT

EXAMPLE 4

Tables A-F

TableA: MVA/48 immunization – guinea pigs.

Groups of guinea pigs were immunized at days 0 and 30 with 1 $x10^8$ infectious units of MVA/48 by either the intramuscular (IM) or intradermal (ID) route. As a control another group was immunized IM with the same dose of non-recombinant MVA. Sera taken before as well as after each immunization was analyzed for neutralizing activity against HIV-1-MN. Titers are the reciprocal serum dilution at which 50% of MT-2 cells were protected from virus-induced killing. Significant neutralizing activity was observed in all animals after the second immunization with MVA/48 (day 49).

Table B: Frequencies of HIV-1 gag-specific T cells following immunization of mice with MVA/48.

Groups of BalbC mice were immunized at days 0 and 21 with 1 $x10^7$ infectious units of MVA/48 by one of three routes: intraperitoneal (IP), intradermal (ID), or intramuscular (IM). A control group was immunized with non-recombinant MVA. At 5 weeks after the last immunization, splenocytes were prepared and stimulated *in vitro* with an immunodominant peptide from HIV-1 p24 for 7 days. The cells were then mixed either with peptide-pulsed P815 cells or with soluble peptide. Gamma interferon-producing cells were enumerated in an ELISPOT assay. A value of >500 was assigned to wells containing too many spots to count. Strong T cell responses have been reported in mice immunized IP

with other viruses. In this experiment, IP immunization of mice with MVA/48 elicited very strong HIV-1 gag-specific T cell responses.

Table C: DNA prime and MVA/48 boost – total ELISPOTS per animal.

Ten rhesus macaques were primed (weeks 0 and 8) with a DNA vaccine expressing HIV-1 antigens including Ada envelope and HXB2 gagpol. At week 24 the animals were boosted intramuscularly with 1 $x10^8$ infectious units of MVA/48. Fresh peripheral blood mononuclear cells (PBMC) were analyzed for production of gamma interferon in an ELISPOT assay as follows: PBMC were incubated for 30-36 hours in the presence of pools of overlapping peptides corresponding to the individual HIV-1 antigens in the vaccines. The total number of gamma interferon-producing cells from each animal is shown in the table. T cell responses to DNA vaccination were limited (weeks 2-20). However, boosting with MVA/48 resulted in very strong HIV-1-specific T cell responses in all animals (week 25).

Table D: Antibody response following immunization of macaques with MVA/SHIV KB9.

Groups of rhesus macaques were immunized with 2 $x10^8$ infectious units of MVA/SHIV-KB9 at weeks 0 and 4 by one of several routes: Tonsilar, intradermal (ID), or intramuscular (IM). Another group was immunized with non-recombinant MVA using the same routes. Serum samples from 2 weeks after the second immunization were analyzed for binding to KB9 envelope protein by ELISA and for neutralization of SHIV-89.6P and SHIV-89.6. In the ELISA assay, soluble KB9 envelope protein was captured in 96 well plates using an antibody to the C-terminus of gp120. Serial dilutions of sera were analyzed and used to determine the endpoint titers. Neutralization of SHIV-89.6P and SHIV-89.6 was determined in an MT-2 cell assay. Titers are the reciprocal serum dilution at which 50% of the cells were protected from virus-induced killing. In *in vitro* neutralization assays, SHIV-89.6P and SHIV-89.6 are heterologous, i.e. sera from animals infected with one of the viruses does not neutralize the other virus. Thus, two immunizations with MVA/SHIV-KB9 elicited good ELISA binding antibodies in all animals and neutralizing antibodies to the homologous virus (SHIV-89.6P) in some animals. In addition, heterologous neutralizing antibodies were observed in a subset of animals.

Table E: Frequencies of gag CM-9-specific CD3/CD8 T cells following immunization of macaques with MVA/SHIV-KB9.

Groups of MamuA*01 positive rhesus macaques were immunized with 2 $x10^8$ infectious units of MVA/SHIV-KB9 at weeks 0 and 4 by one of several routes: tonsilar, intradermal (ID), or intramuscular IM). Another group was immunized with non-recombinant MVA. The frequencies of CD3+/CD8+ T cells that bound tetrameric complex containing the SIV gag-specific peptide CM9 were determined by flow cytometry at various times after each immunization. Time intervals were as follows: 1a, 1b, and 1d were one, two, and four weeks after the first immunization, respectively; 2a, 2b, 2c, and 2d were one, two, three, and twelve weeks after the second immunization, respectively. Values above background are shown in bold face. Strong SIV gag-specific responses were observed after a single immunization with MVA/SHIV-KB9 in all immunized animals. Boosting was observed in most animals following the second immunization. In addition, measurable tetramer binding was still found twelve weeks after the second immunization.

Table F: Frequencies of specific T cells following immunization of macaques with MVA/SHIV KB9.

Groups of macaques were immunized with MVA/SHIV-KB9 as described above. MVA/SHIV-KB9 expresses 5 genes from the chimeric virus, SHIV-89.6P: envelope, gag, polymerase, tat, and nef. Thus, the frequencies of T cells specific for each of the 5 antigens was analyzed using pools of peptides corresponding to each individual protein. Fresh PBMC were stimulated with pools of peptides for 30-36 hours in vitro. Gamma interferon-producing cells were enumerated in an ELISPOT assay. The total number of cells specific for each antigen is given as "total # spots". In addition, the number of responding animals and average # of spots per group is shown. PBMC were analyzed at one week after the first immunization (1a) and one week after the second immunization (2a). Another group of 7 animals was immunized with non-recombinant MVA. In these animals, no spots above background levels were detected. Thus, a single immunization with MVA/SHIV-KB9 elicited strong SHIV-specific T cell responses in all animals. Gag and envelope responses were the strongest; most animals had responses to gag, all animals had responses to envelope. The Elispot responses were also observed after the second immunization with MVA/SHIV-KB9, albeit at lower levels. At both times, the rank order of responses was: tonsilar > ID > IM. We show good immune response to nef and some immune response to tat.

TABLE A

MVA/48 immunization – guinea pigs
HIV-MN neutralizing antibody - reciprocal titer

Animal #	Group	Route	day 0	Day 4 MVA #1	day 30	day 33 MVA#2	day 49
885	MVA	I.M.	<20	I.M.	31	I.M.	24
891	"	"	<20	"	85	"	<20
882	MVA/48	I.M.	<20	I.M.	<20	I.M.	5,524
883	"	"	<20	"	68	"	691
886	"	"	<20	"	<20	"	4,249
890	"	"	<20	"	180	"	89
879	MVA/48	I.D.	<20	I.D.	<20	I.D.	817
881	"	"	<20	"	<20	"	234
888	"	"	<20	"	24	"	112
889	"	"	<20	"	22	"	376

TABLE B

Frequencies of HIV-gag-specific T cells following immunization of mice with MVA/48

Group	P815 cells + gag peptide		gag peptide		no stimulation	
MVA control	0	2	0	4	1	2
MVA/48 (IP)	>500	>500	>500	>500	8	8
MVA/48 (ID)	12	5	49	33	4	2
MVA/48 (IM)	22	18	66	49	12	8

TABLE C

DNA prime and MVA/48 boost
Total ELISPOTS per Animal

Animal #	WEEKS						
	-2	2	6	10[2]	14[2]	20[2]	25[2]
RLw	4	731*	<	47	43	50	3905
RVl	5	997*	<	<	<	8	205
Roa	<[1]	<	1	<	<	<	245
RHc	<	<	<	<	<	<	535
Ryl	<	<	<	<	<	<	4130
RQk	<	46	<	<	<	<	630
RDr	<	<	<	14	<	<	1965
RZc	<	5	<	58	<	<	925
RSf	<	118	<	<	<	20	5570
Ras	<	69	<	<	<	<	1435
Total	9	1966	1	119	43	78	19545
Geo Mean	4.5	105.3	1.0	33.7	43.0	20.0	1147.7

DNA primes were at 0 and 8 weeks and MVA/48 boost was at 24 weeks

[1] < = Background (2x the number of ELISPOTs in the unstimulated control + 10)
[2]Costimulatory antibodies were added to the ELISPOT incubations
* Animals from this bleed date exhibited higher than usual ELISPOTs.

TABLE D

Antibody response following immunization of macaques with MVA/SHIV KB9

Animal #	Route	KB9 env ELISA titer	KB9 env elisa average	KB9 env elisa std dev.	SHIV-89.6 Nab titer	SHIV-89.6P Nab titer	SHIV-89.6 # pos animals	SHIV-89.6P # pos animals
598	tonsil	25,600	31,086	20,383	<20	<20	3	2
601	"	51,200			<20	<20		
606	"	25,600			<20	<20		
642	"	51,200			75	31		
646	"	51,200			61	48		
653	"	6,400			<20	<20		
654	"	6,400			22	<20		
602	i.d.	25,600	18,800	15,341	38	<20	2	4
604	"	12,800			<20	262		
608	"	3,200			20	66		
637	"	12,800			<20	35		
638	"	51,200			<20	<20		
645	"	25,600			<20	<20		
647	"	12,800			32	162		
650	"	6,400			<20	<20		
599	i.m.	6,400	17,000	16,516	<20	<20	0	3
600	"	6,400			<20	29		
609	"	6,400			<20	<20		
639	"	51,200			<20	85		
640	"	12,800			<20	<20		
641	"	25,600			<20	41		
649	"	1,600			<20	<20		
651	"	25,600			20	<20		
603	Control	<100	<100		<20	<20	0	0
605	"	<100			<20	<20		
607	"	<100			<20	<20		
643	"	<100			<20	<20		
644	"	<100			<20	<20		
648	"	<100			<20	<20		
652	"	<100			<20	<20		

TABLE E

Frequencies of gag CM9-specific CD3/CD8 T cells following immunization of macaques with MVA/SHIV KB9

Animal #	Route	Virus	pre-bleed	1a	1b	1d	2a	2b	2c	2d
598	Tonsil	MVA/KB9	0.018	**0.41**	**0.79**	**0.25**	**2.64**	**1.13**	**0.51**	**0.21**
601	"	"	0.071	**0.34**	**0.38**	**0.27**	**0.83**	**0.7**	**0.36**	0.039
646	"	"	0.022	**0.68**	**0.76**	**0.43**	**1.12**	**0.91**	**0.53**	**0.15**
653	"	"	0.041	**0.69**	**0.85**	**0.53**	**0.68**	**0.49**	**0.47**	**0.3**
648	"	MVA		0.033	0.039		0.022	0.058	0.033	0.013
602	i.d.	MVA/KB9	0.019	**0.17**	**0.92**	**0.5**	**0.95**	**0.59**	**0.5**	**0.2**
604	"	"	0.013	**0.11**	**0.38**	**0.32**	**0.44**	**0.38**	**0.19**	**0.25**
650	"	"	0.095	**0.17**	**0.6**	**0.23**	**2.87**	**1.12**	**0.9**	**0.16**
647	"	"	0.032	**0.22**	**0.38**	**0.14**	**0.84**	**0.91**	**0.34**	**0.17**
652	"	MVA		0.041	0.038	0.059	0.025	0.022	0.026	0.055
599	i.m.	MVA/KB9		0.081	**0.31**	0.082		0.12	0.054	0.11
600	"	"	0.034	**0.15**	**0.41**	**0.17**	**0.29**	**0.27**	**0.16**	0.049
649	"	"	0.00486	**0.35**	**1.34**	**0.56**	**2.42**	**0.77**	**0.69**	0.22
651	"	"	0.049	**0.12**	**0.69**	**0.25**	**1.01**	**0.32**	**0.24**	0.22
603	"	MVA		0.024	0.087	0.073		0.082	0.027	0.17

TABLE F

Frequencies of specific T cells following immunization of macaques with MVA/SHIV KB9

	Gag specific			Tat specific			Nef specific			Env specific			Total
Study groups	# responding animals	Total # spots	average # spots	# responding animals	total # spots	average # spots	# responding animals	total # spots	average # spots	# responding animals	total spots	Average # spots	# responding animals
tonsil 1a	4/6	1325	221	0/6	0	0	3/6	195	33	6/6	8760	1460	6/6
tonsil 2a	5/6	1405	234	0/6	0	0	1/6	560	93	6/6	4485	748	6/6
i.d. 1a	7/7	1335	191	0/7	0	0	2/7	215	31	7/7	7320	1046	7/7
i.d. 2a	4/7	755	108	0/7	0	0	1/7	55	8	7/7	2700	386	7/7
i.m. 1a	7/7	925	132	1/7	60	9	3/7	180	26	7/7	5490	784	7/7
i.m. 2a	4/7	250	36	0/7	0	0	0/7	0	0	6/7	2205	315	6/7

*****

While the present invention has been described in some detail for purposes of clarity and understanding, one skilled in the art will appreciate that various changes in form and detail can be made without departing from the true scope of the invention. All patents, patent applications and publications referred to above are hereby incorporated by reference.

WHAT IS CLAIMED IS:

1. A pharmaceutical composition comprising a recombinant MVA virus expressing an HIV *env*, *gag*, and *pol* gene or modified gene thereof for production of an HIV Env, Gag, and Pol antigen by expression from said recombinant MVA virus, wherein said HIV *env* gene is modified to encode an HIV Env protein composed of gp120 and the membrane-spanning and ectodomain of gp41 but lacking part or all of the cytoplasmic domain of gp41, and a pharmaceutically acceptable carrier.

2. The pharmaceutical composition of claim 1, wherein said HIV *pol* gene or modified gene thereof is modified to inactivate reverse transcriptase and integrase.

3. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade A.

4. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade B.

5. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade C.

6. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade D.

7. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade E.

8. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade F.

9. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade G.

10. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade H.

11. The pharmaceutical composition of claim 1, wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is taken from clade J.

12. The pharmaceutical composition of claim 1 wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is inserted at the site of deletion III within the MVA genome.

13. The pharmaceutical composition of claim 1 wherein said HIV *env*, *gag*, or *pol* gene or modified gene thereof is under transcriptional initiation regulation of a H5-like early/late vaccinia virus promoter.

14. The pharmaceutical composition of claim 1 wherein recombinant MVA virus additionally expresses an additional HIV gene or modified gene thereof for production of an HIV antigen by expression from said recombinant MVA virus, wherein said additional HIV gene is a member selected from the group consisting of *vif, vpr, tat, rev, vpu*, and *nef*.

15. MVA/HIV48 comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, and SEQ ID NO:5.

16. pLW-48 having SEQ ID NO:1.

17. A plasmid transfer vector having the sequence of pLW-48 (SEQ ID NO:1) excluding the HIV *env*, *gag*, and *pol* genes.

18. pLW-48 (SEQ ID NO:1) wherein the HIV *env*, *gag*, and *pol* genes have a sequence taken from another clade.

19. A poxvirus comprising a promoter selected from the group consisting of m7.5 promoter having SEQ ID NO:10, Psyn II promoter having SEQ ID NO:2, Psyn III promoter having SEQ ID NO:11, Psyn IV promoter having SEQ ID NO:12, and Psyn V promoter having SEQ ID NO:13.

20. A method of boosting a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a composition of any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen previously primed in the primate is boosted.

21. A method of inducing a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a composition of any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen in the primate is induced.

22. A method of inducing a $CD8^+$ T cell immune response to an HIV Env, Gag, or Pol antigen in a primate, the method comprising provision in the primate of a priming composition comprising nucleic acid encoding said antigen and then provision in the primate of a boosting composition which comprises any of claims 1-15, whereby a $CD8^+$ T cell immune response to the antigen is induced.

23. The method of any of Claims 20-22, wherein the primate is a human.

24. The method of any of Claims 20-22, wherein administration of the combinant MVA virus is by needleless injection.

25. The method of Claim 22, wherein the priming composition comprises mid DNA encoding said antigen.

26. A method of making a composition of any of claims 1-15 comprising paring a plasmid transfer vector encoding an HIV *env*, *gag*, and *pol* gene or modified e thereof, wherein said HIV *env* gene is modified to encode an HIV Env protein posed of gp120 and the membrane-spanning and ectodomain of gp41 but lacking part all of the cytoplasmic domain of gp41, and recombining said plasmid transfer vector a MVA virus to produce a composition of any of claims 1-15.

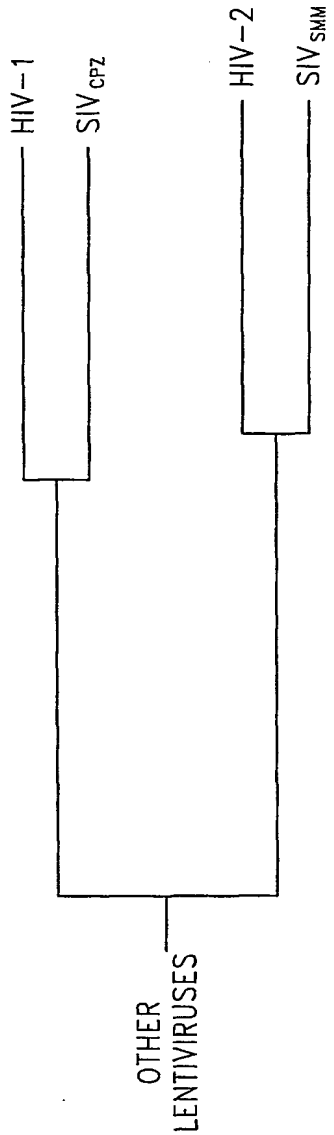

FIG. 1

(Figure I)

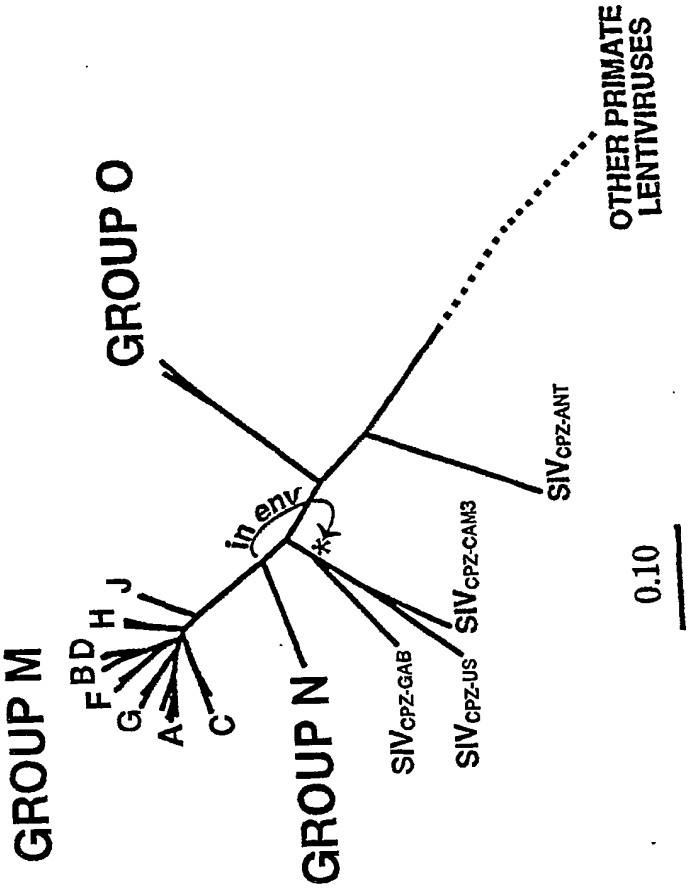

FIG. 2

(Figure II)

Chemokine coreceptor used	PBMC replication	Macrophage replication	T-cell-line replication	REplicative phenotype	Syncytium-inducing phenotype
X4	+	-	+	Rapid/high	++
R5	+	+	-	Slow/low	-
R5/X4	+	+	+	Rapid/high	+

FIG. 3

(Figure III)

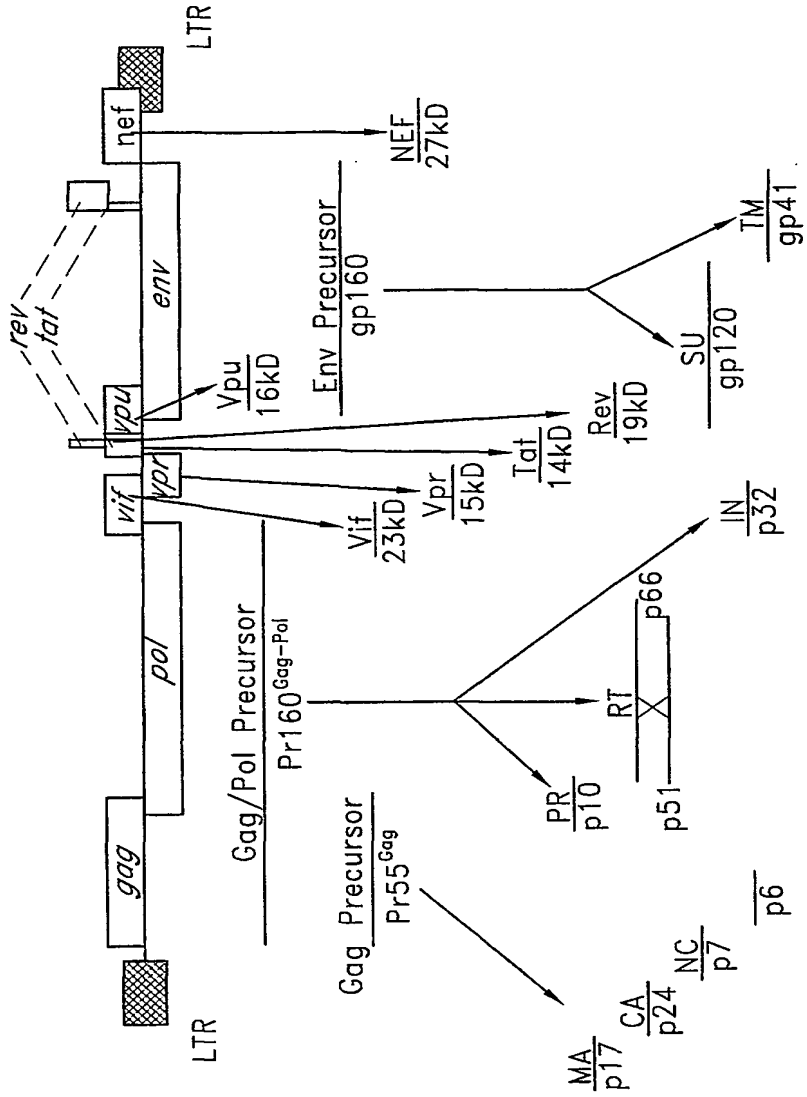

FIG. 4

(Figure IV)

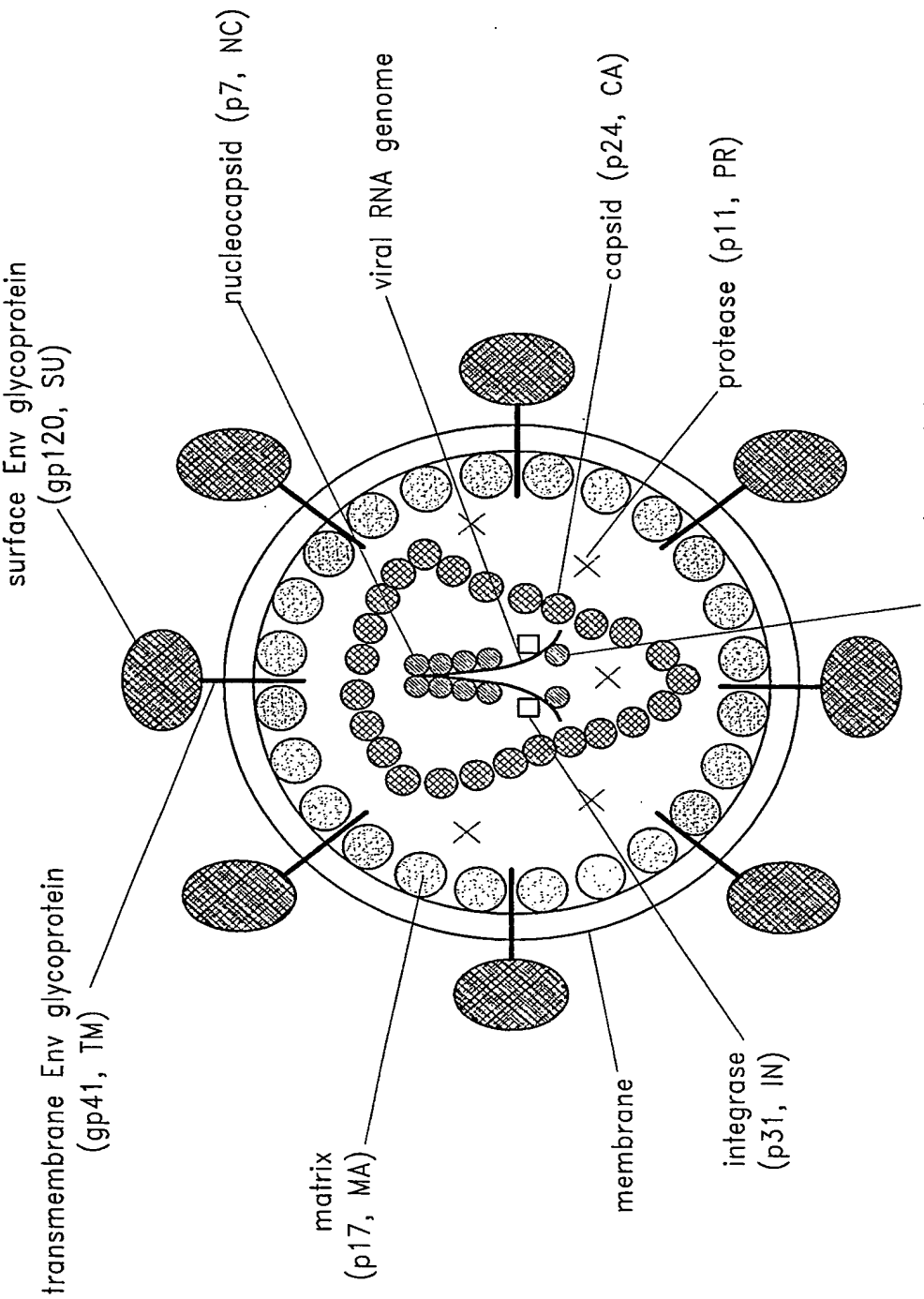

FIG. 5

(Figure V)

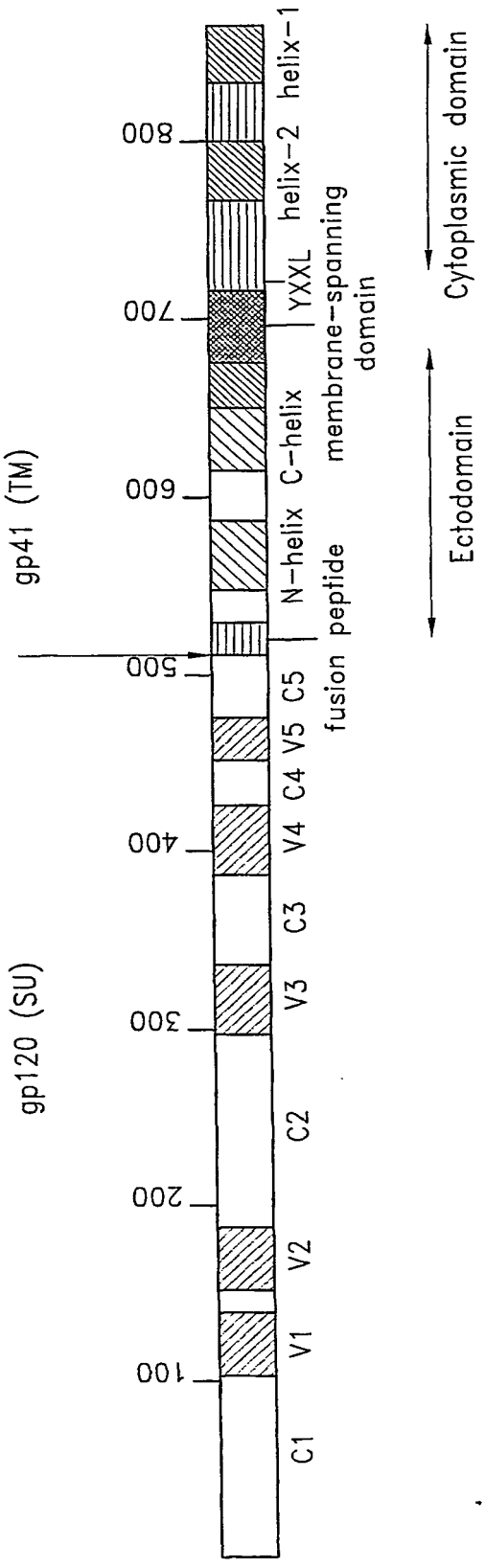

FIG. 6

(Figure VI)

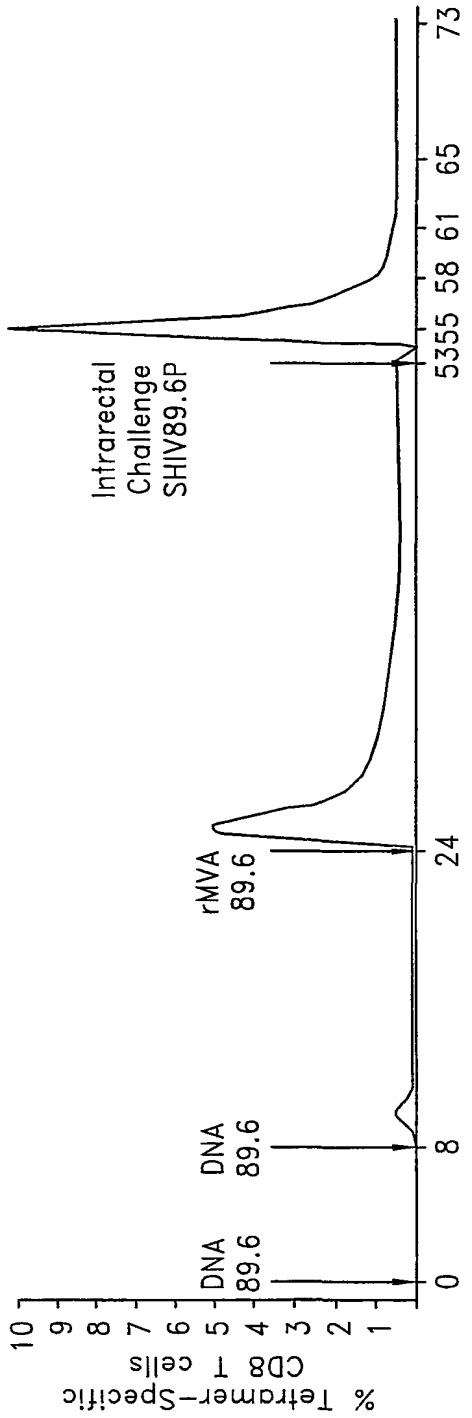

FIG. 7A
(Figure 1A)

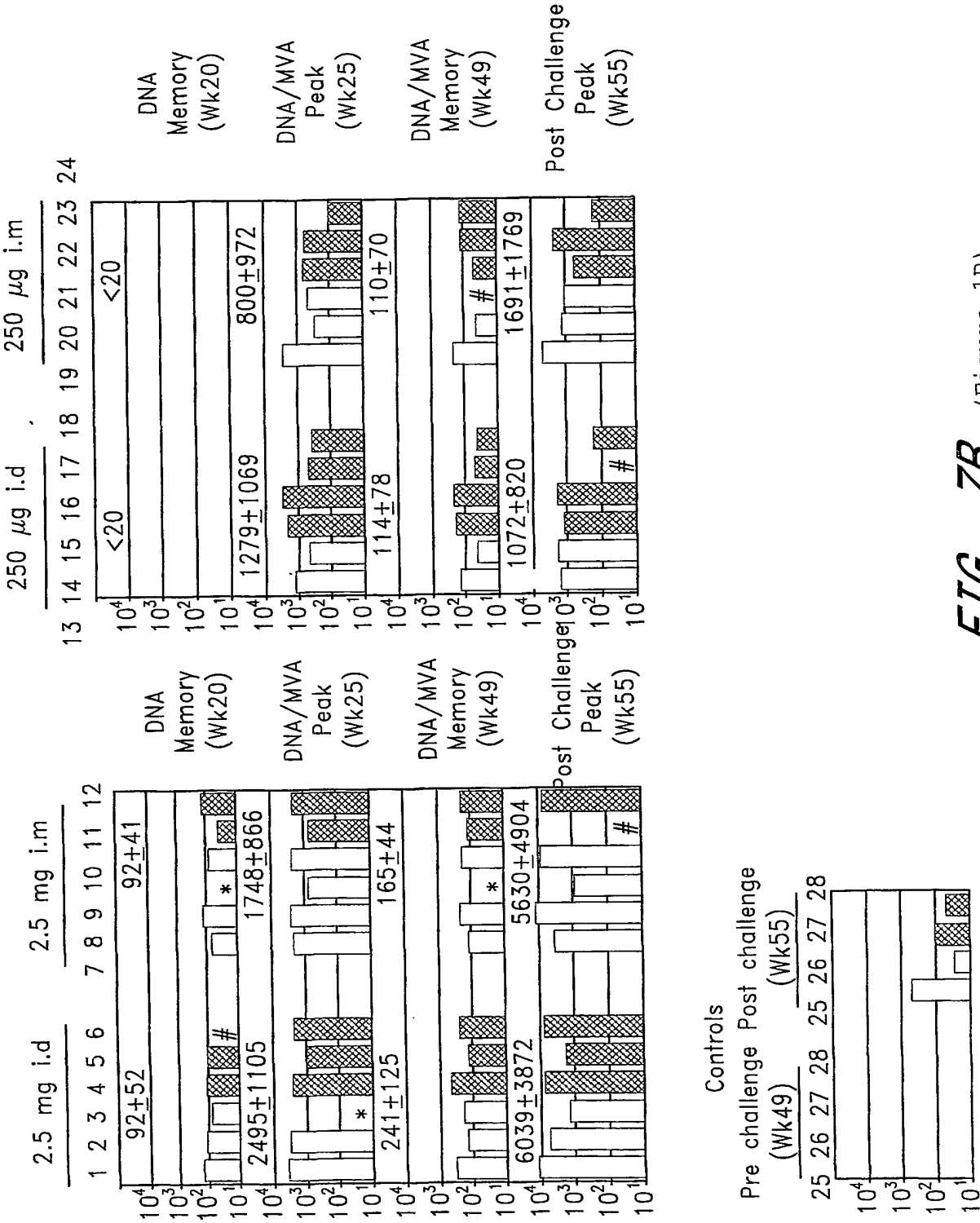

FIG. 7B (Figure 1B)

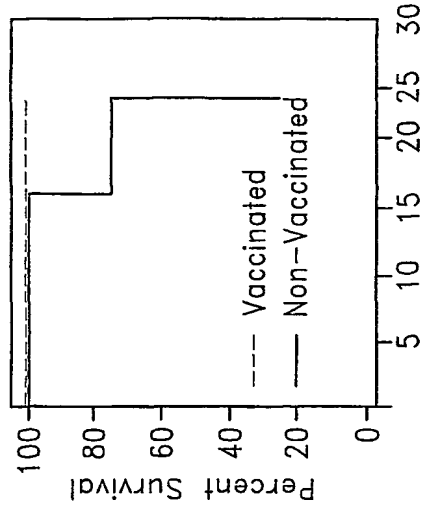

FIG. 8C (Figure 2C)

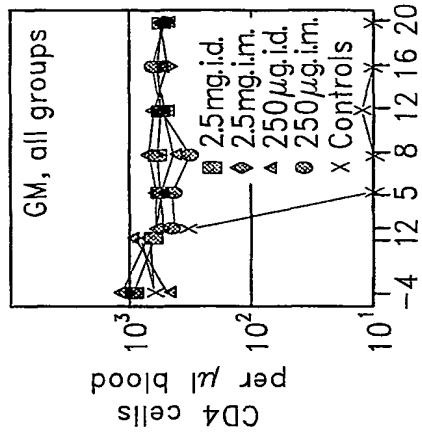

FIG. 8B (Figure 2B)

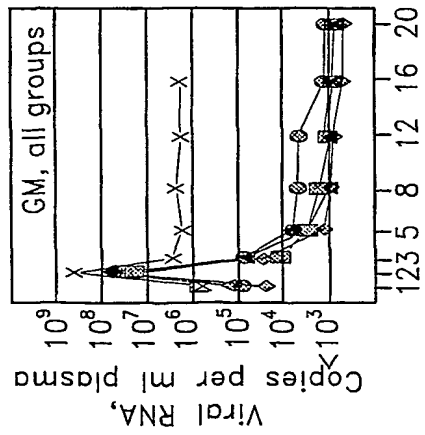

FIG. 8A (Figure 2A)

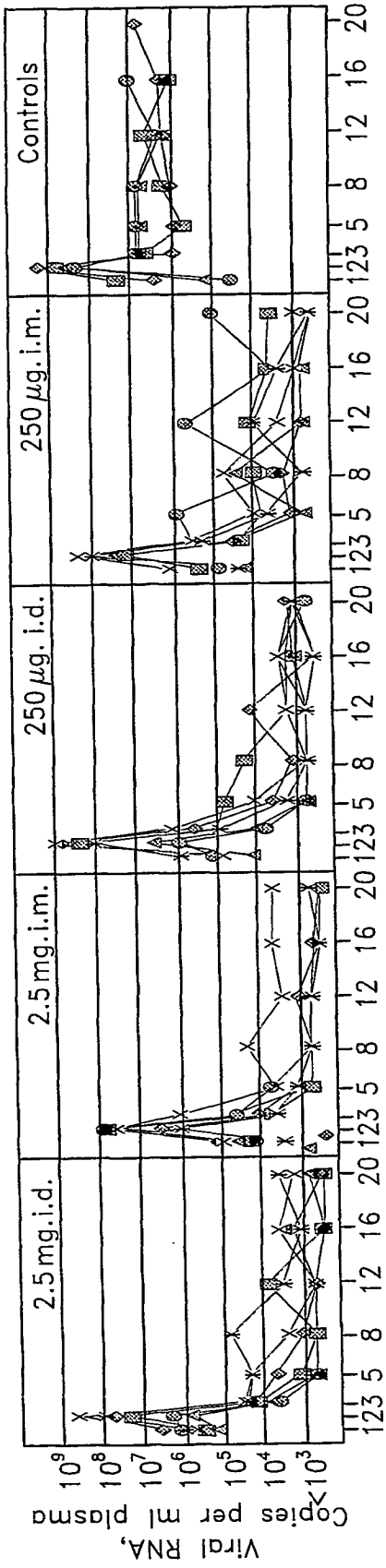

FIG. 8D
(Figure 2D)

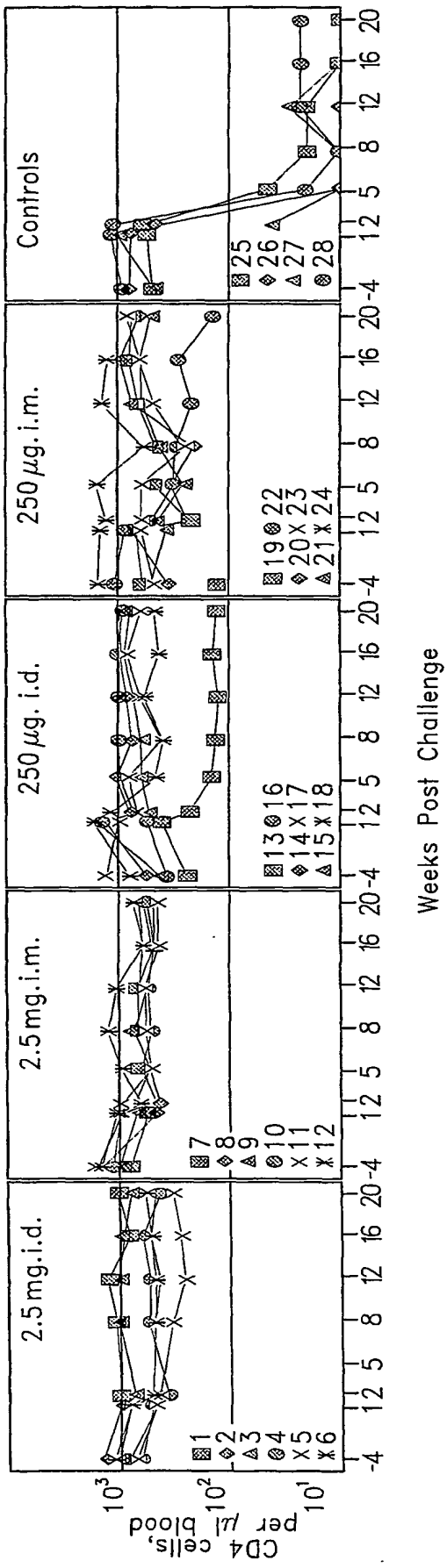

FIG. 8E
(Figure 2E)

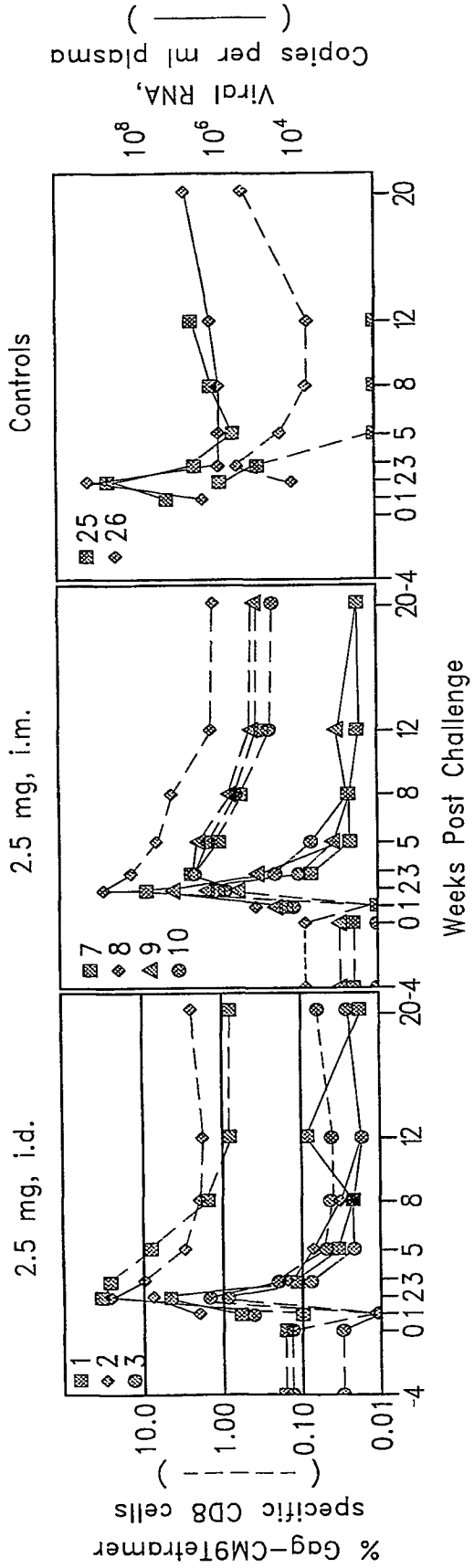

FIG. 9A
(Figure 3A)

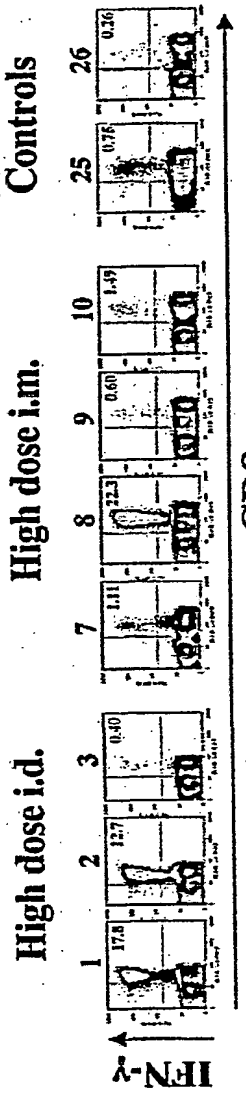

FIG. 9B

(Figure 3B)

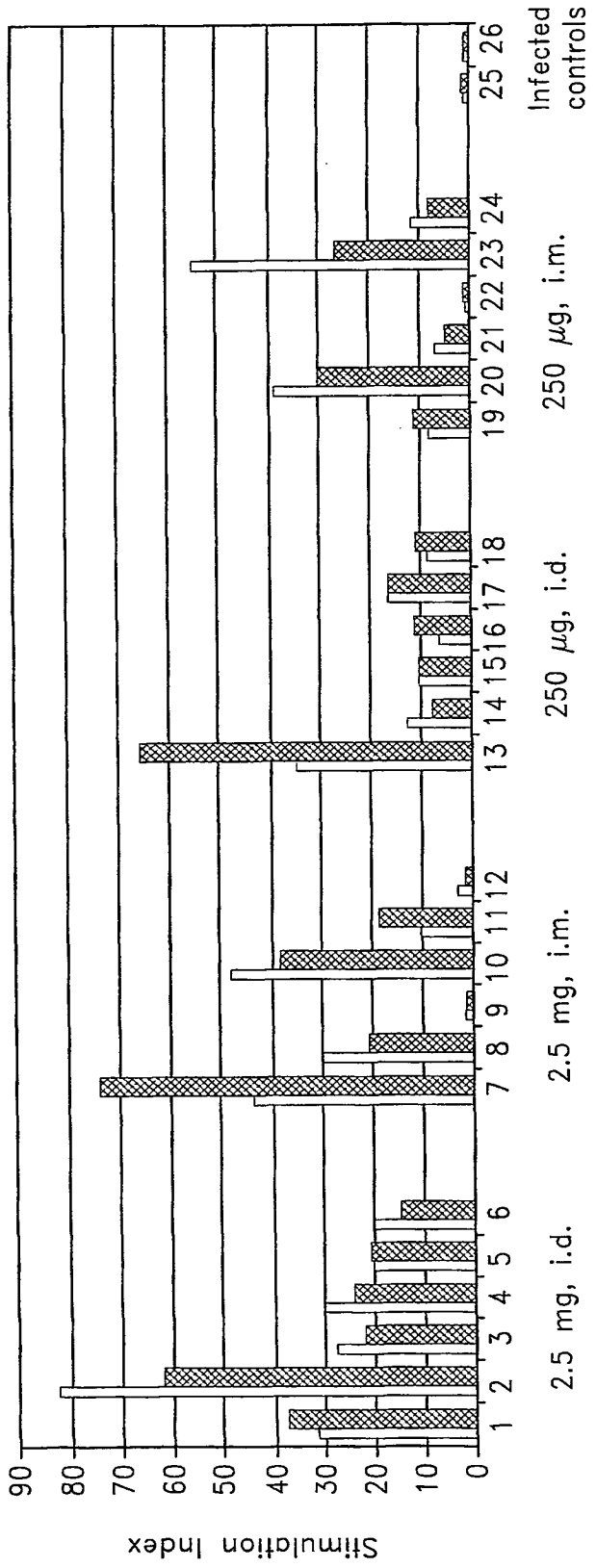

FIG. 9C

(Figure 3C)

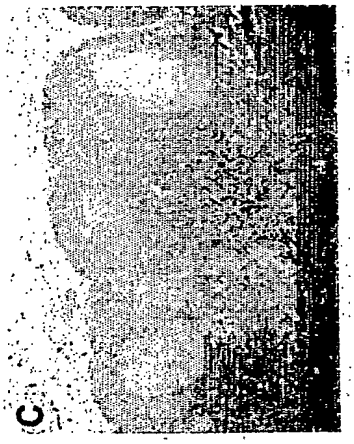

FIG. 10C (Figure 4C)

FIG. 10B (Figure 4B)

FIG. 10A (Figure 4A)

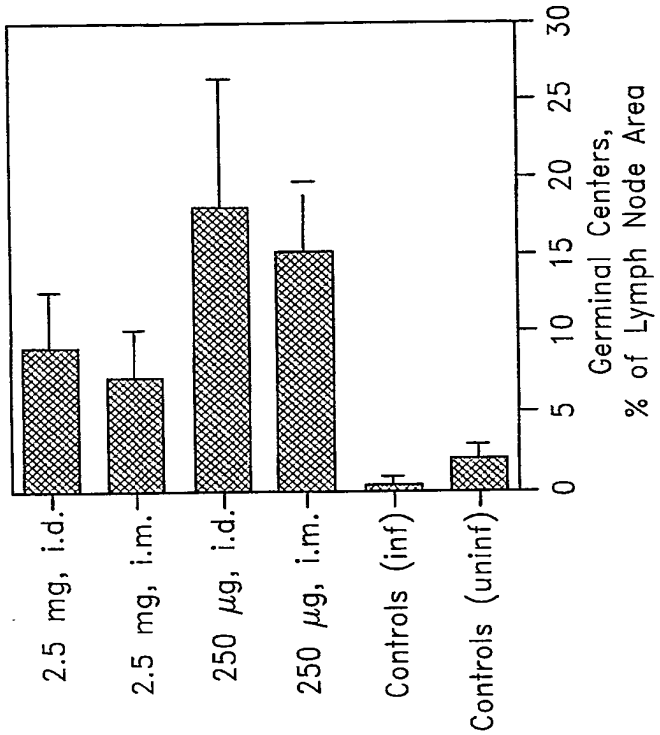

FIG. 10D

(Figure 4D)

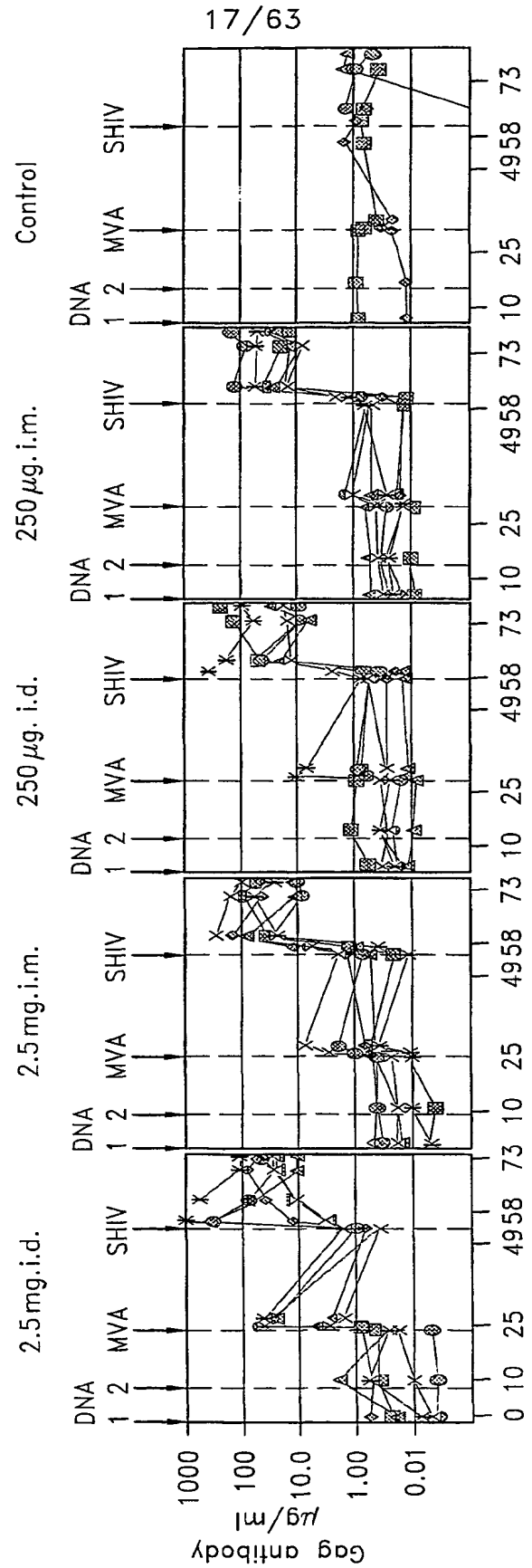

FIG. 11A

(Figure 5A)

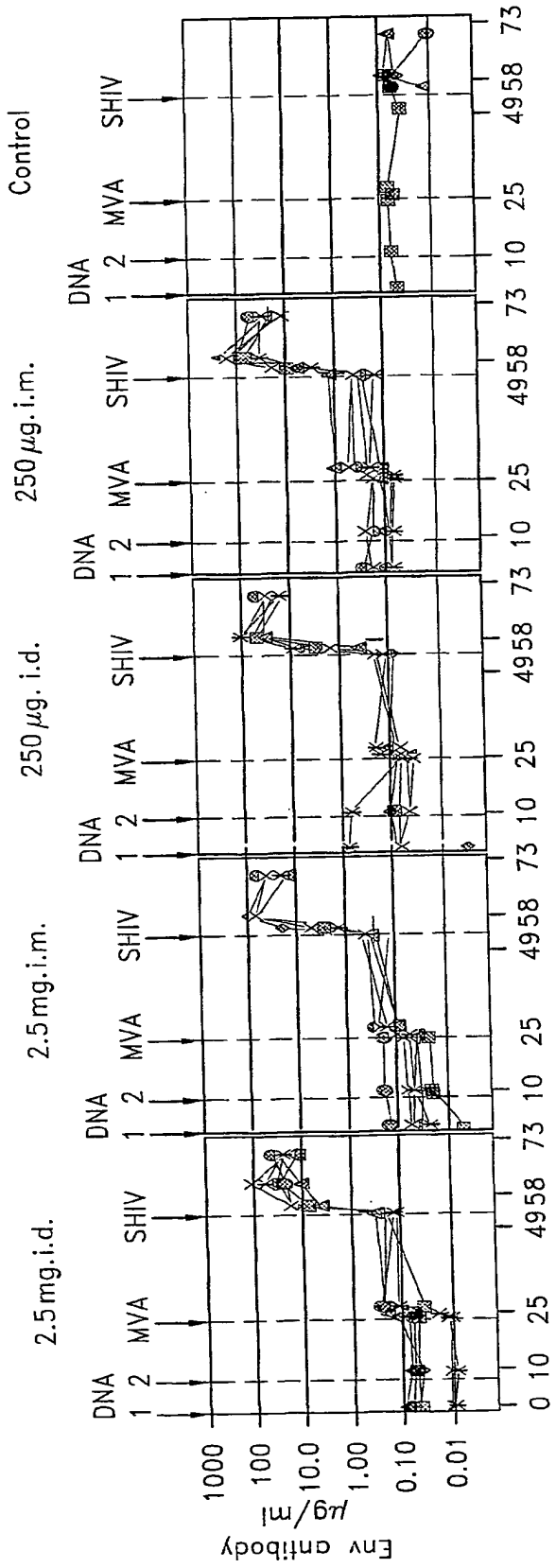

FIG. 11B

(Figure 5B)

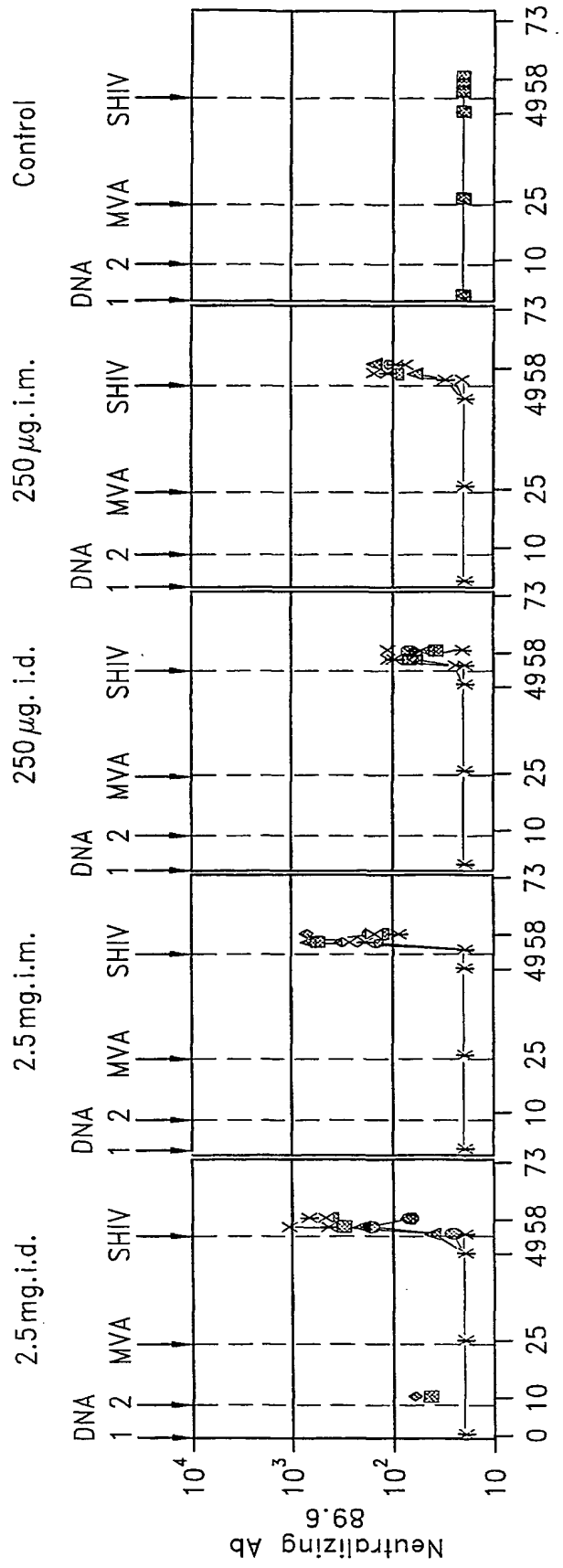

FIG. 11C

(Figure 5C)

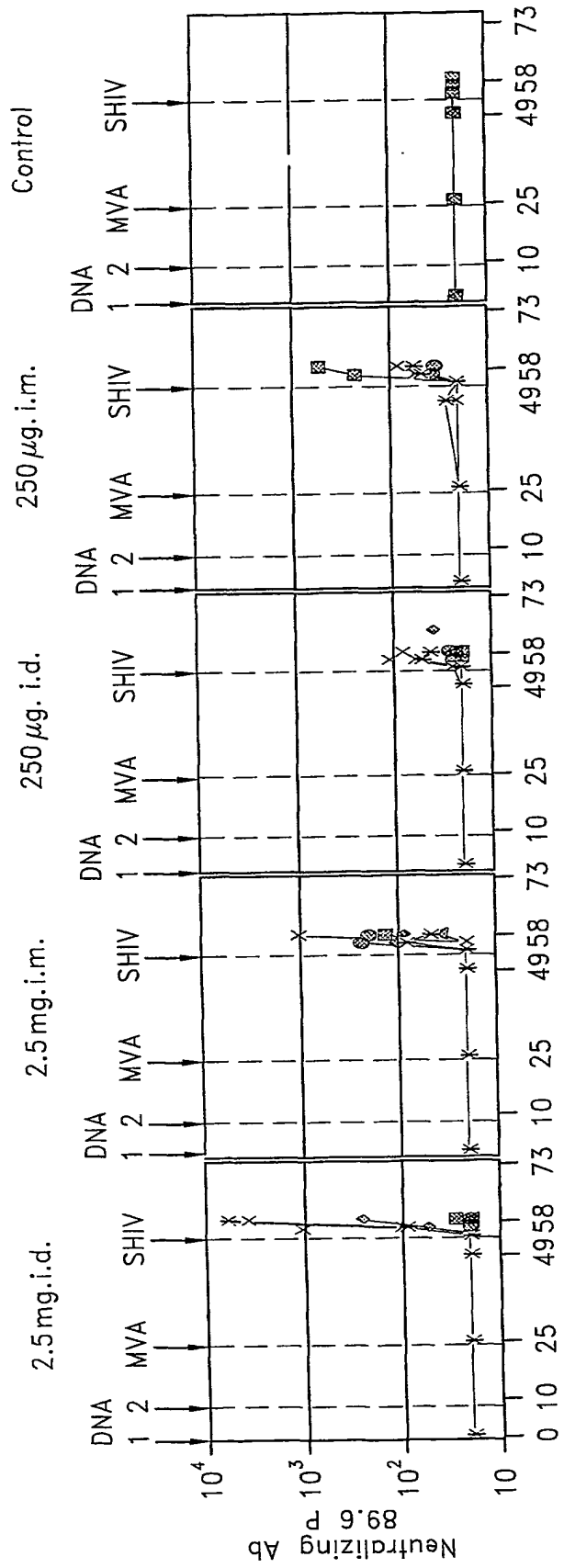

FIG. 11D

(Figure 5D)

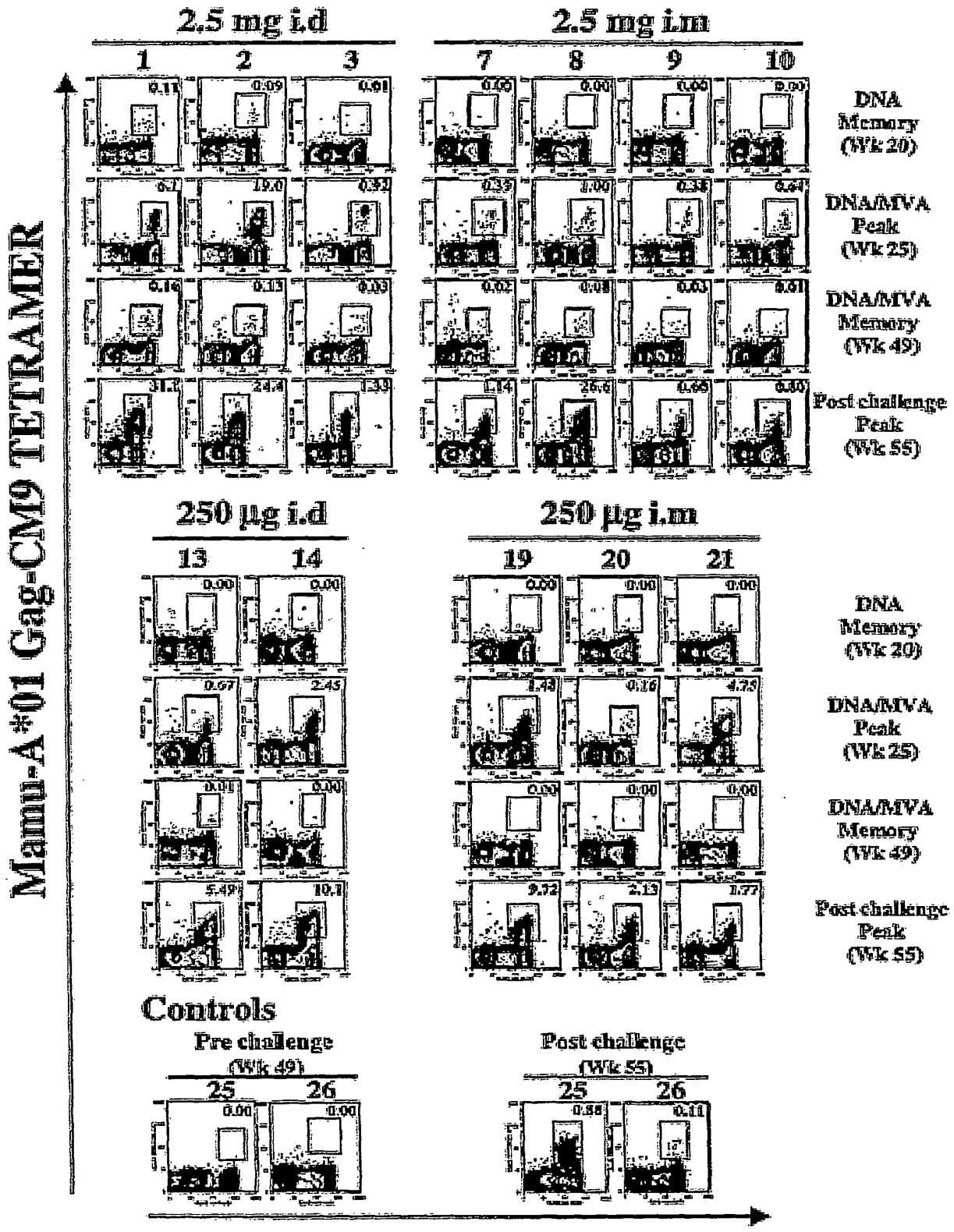

FIG. 12

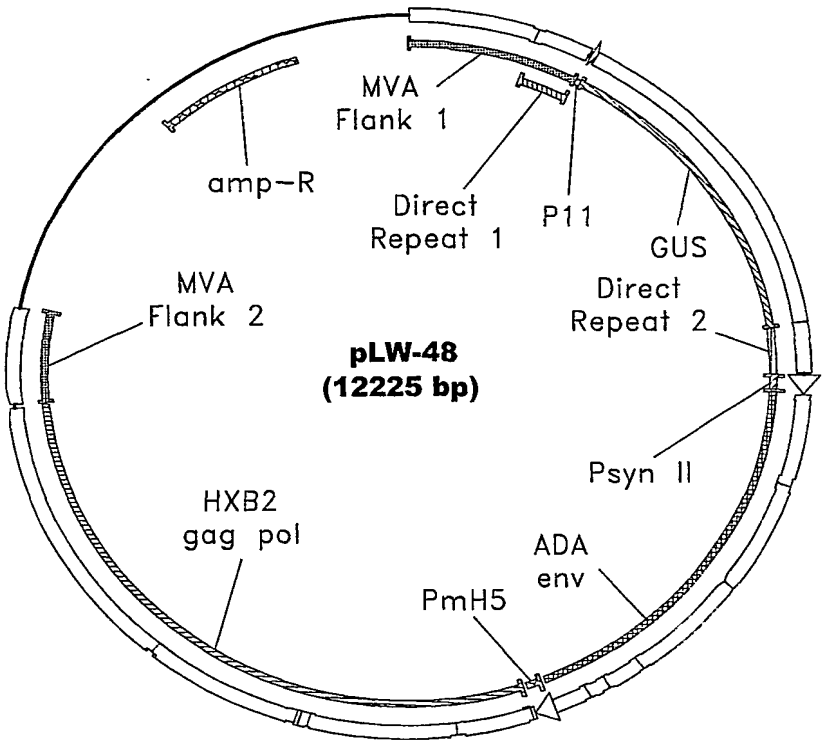

FIG. 13

(FIG. A_1)

```
  1 GAATTCGTTG GTGGTCGCCA TGGATGGTGT TATTGTATAC TGTCTAAACG CGTTAGTAAA ACATGGCGAG
    CTTAAGCAAC CACCAGCGGT ACCTACCACA ATAACATATG ACAGATTTGC GCAATCATTT TGTACCGCTC

 71 GAAATAAATC ATATAAAAAA TGATTTCATG ATTAAACCAT GTTGTGAAAA AGTCAAGAAC GTTCACATTG
    CTTTATTTAG TATATTTTTT ACTAAAGTAC TAATTTGGTA CAACACTTTT TCAGTTCTTG CAAGTGTAAC

141 GCGGACAATC TAAAAACAAT ACAGTGATTG CAGATTTGCC ATATATGGAT AATGCGGTAT CCGATGTATG
    CGCCTGTTAG ATTTTTGTTA TGTCACTAAC GTCTAAACGG TATATACCTA TTACGCCATA GGCTACATAC

211 CAATTCACTG TATAAAAAGA ATGTATCAAG AATATCCAGA TTTGCTAATT TGATAAAGAT AGATGACGAT
    GTTAAGTGAC ATATTTTTCT TACATAGTTC TTATAGGTCT AAACGATTAA ACTATTTCTA TCTACTGCTA

281 GACAAGACTC CTACTGGTGT ATATAATTAT TTTAAACCTA AAGATGCCAT TCCTGTTATT ATATCCATAG
    CTGTTCTGAG GATGACCACA TATATTAATA AAATTTGGAT TTCTACGGTA AGGACAATAA TATAGGTATC

351 GAAAGGATAG AGATGTTTGT GAACTATTAA TCTCATCTGA TAAAGCGTGT GCGTGTATAG AGTTAAATTC
    CTTTCCTATC TCTACAAACA CTTGATAATT AGAGTAGACT ATTTCGCACA CGCACATATC TCAATTTAAG
```

FIG. 14 (FIG. A_2)

421 ATATAAAGTA GCCATTCTTC CCATGGATGT TTCCTTTTTT ACCAAAGGAA ATGCATCATT GATTATTCTC
TATATTTCAT CGGTAAGAAG GGTACCTACA AAGGAAAAAA TGGTTTCCTT TACGTAGTAA CTAATAAGAG

491 CTGTTTGATT TCTCTATCGA TGCGGCACCT CTCTTAAGAA GTGTAACCGA TAATAATGTT ATTATATCTA
GACAAACTAA AGAGATAGCT ACGCCGTGGA GAGAATTCTT CACATTGGCT ATTATTACAA TAATATAGAT

561 GACACCAGCG TCTACATGAC GAGCTTCCGA GTTCCAATTG GTTCAAGTTT TACATAAGTA TAAAGTCCGA
CTGTGGTCGC AGATGTACTG CTCGAAGGCT CAAGGTTAAC CAAGTTCAAA ATGTATTCAT ATTTCAGGCT

631 CTATTGTTCT ATATTATATA TGGTTGTTGA TGGATCTGTG ATGCATGCAA TAGCTGATAA TAGAACTTAC
GATAACAAGA TATAATATAT ACCAACAACT ACCTAGACAC TACGTACGTT ATCGACTATT ATCTTGAATG

701 GCAAATATTA GCAAAAATAT ATTAGACAAT ACTACAATTA ACGATGAGTG TAGATGCTGT TATTTTGAAC
CGTTTATAAT CGTTTTTATA TAATCTGTTA TGATGTTAAT TGCTACTCAC ATCTACGACA ATAAAACTTG

FIG. 15

(FIG. $A_{2\ cont.}$)

771 CACAGATTAG GATTCTTGAT AGAGATGAGA TGCTCAATGG ATCATCGTGT GATATGAACA GACATTGTAT
GTGTCTAATC CTAAGAACTA TCTCTACTCT ACGAGTTACC TAGTAGCACA CTATACTTGT CTGTAACATA

841 TATGATGAAT TTACCTGATG TAGGCGAATT TGGATCTAGT ATGTTGGGGA AATATGAACC TGACATGATT
ATACTACTTA AATGGACTAC ATCCGCTTAA ACCTAGATCA TACAACCCCT TTATACTTGG ACTGTACTAA

911 AAGATTGCTC TTTCGGTGGC TGGGTACCAG GCGCGCCTTT CATTTTGTTT TTTTCTATGC TATAAATGGT
TTCTAACGAG AAAGCCACCG ACCCATGGTC CGCGCGGAAA GTAAAACAAA AAAAGATACG ATATTTACCA

981 ACGTCCTGTA GAAACCCCAA CCCGTGAAAT CAAAAAACTC GACGGCCTGT GGGCATTCAG TCTGGATCGC
TGCAGGACAT CTTTGGGGTT GGGCACTTTA GTTTTTTGAG CTGCCGGACA CCCGTAAGTC AGACCTAGCG

1051 GAAAACTGTG GAATTGATCA GCGTTGGTGG GAAAGCGCGT TACAAGAAAG CCGGGCAATT GCTGTGCCAG
CTTTTGACAC CTTAACTAGT CGCAACCACC CTTTCGCGCA ATGTTCTTTC GGCCCGTTAA CGACACGGTC

FIG. 16

(FIG. A$_{2\ cont.}$)

```
1121 GCAGTTTTAA CGATCAGTTC GCCGATGCAG ATATTCGTAA TTATGCGGGC AACGTCTGGT ATCAGCGCGA
     CGTCAAAATT GCTAGTCAAG CGGCTACGTC TATAAGCATT AATACGCCCG TTGCAGACCA TAGTCGCGCT

1191 AGTCTTTATA CCGAAAGGTT GGGCAGGCCA GCGTATCGTG CTGCGTTTCG ATGCGGTCAC TCATTACGGC
     TCAGAAATAT GGCTTTCCAA CCCGTCCGGT CGCATAGCAC GACGCAAAGC TACGCCAGTG AGTAATGCCG

1261 AAAGTGTGGG TCAATAATCA GGAAGTGATG GAGCATCAGG GCGGCTATAC GCCATTTGAA GCCGATGTCA
     TTTCACACCC AGTTATTAGT CCTTCACTAC CTCGTAGTCC CGCCGATATG CGGTAAACTT CGGCTACAGT

1331 CGCCCGTATG TATTGCCGGG AAAAGTGTAC GTATCACCGT TTGTGTGAAC AACGAACTGA ACTGGCAGAC
     GCGGGCATAC ATAACGGCCC TTTTCACATG CATAGTGGCA AACACACTTG TTGCTTGACT TGACCGTCTG

1401 TATCCCGCCG GGAATGGTGA TTACCGACGA AAACGGCAAG AAAAAGCAGT CTTACTTCCA TGATTTCTTT
     ATAGGGCGGC CCTTACCACT AATGGCTGCT TTTGCCGTTC TTTTTCGTCA GAATGAAGGT ACTAAAGAAA

1471 AACTATGCCG GAATCCATCG CAGCGTAATG CTCTACACCA CGCCGAACAC CTGGGTGGAC GATATCACCG
     TTGATACGGC CTTAGGTAGC GTCGCATTAC GAGATGTGGT GCGGCTTGTG GACCCACCTG CTATAGTGGC
```

FIG. 17 (FIG. $A_{2\ cont.}$)

```
1541 TGGTGACGCA TGTCGCGCAA GACTGTAACC ACGCGTCTGT TGACTGGCAG GTGGTGGCCA ATGGTGATGT
     ACCACTGCGT ACAGCGCGTT CTGACATTGG TGCGCAGACA ACTGACCGTC CACCACCGGT TACCACTACA

1611 CAGCGTTGAA CTGCGTGATG CGGATCAACA GGTGGTTGCA ACTGGACAAG GCACTAGCGG GACTTTGCAA
     GTCGCAACTT GACGCACTAC GCCTAGTTGT CCACCAACGT TGACCTGTTC CGTGATCGCC CTGAAACGTT

1681 GTGGTGAATC CGCACCTCTG GCAACCGGGT GAAGGTTATC TCTATGAACT GTGCGTCACA GCCAAAAGCC
     CACCACTTAG GCGTGGAGAC CGTTGGCCCA CTTCCAATAG AGATACTTGA CACGCAGTGT CGGTTTTCGG

1751 AGACAGAGTG TGATATCTAC CCGCTTCGCG TCGGCATCCG GTCAGTGGCA GTGAAGGGCG AACAGTTCCT
     TCTGTCTCAC ACTATAGATG GGCGAAGCGC AGCCGTAGGC CAGTCACCGT CACTTCCCGC TTGTCAAGGA

1821 GATTAACCAC AAACCGTTCT ACTTTACTGG CTTTGGTCGT CATGAAGATG CGGACTTGCG TGGCAAAGGA
     CTAATTGGTG TTTGGCAAGA TGAAATGACC GAAACCAGCA GTACTTCTAC GCCTGAACGC ACCGTTTCCT

1891 TTCGATAACG TGCTGATGGT GCACGACCAC GCATTAATGG ACTGGATTGG GGCCAACTCC TACCGTACCT
     AAGCTATTGC ACGACTACCA CGTGCTGGTG CGTAATTACC TGACCTAACC CCGGTTGAGG ATGGCATGGA
```

FIG. 18 (FIG. $A_{2\ cont.}$)

```
1961  CGCATTACCC TTACGCTGAA GAGATGCTCG ACTGGGCAGA TGAACATGGC ATCGTGGTGA TTGATGAAAC
      GCGTAATGGG AATGCGACTT CTCTACGAGC TGACCCGTCT ACTTGTACCG TAGCACCACT AACTACTTTG

2031  TGCTGCTGTC GGCTTTAACC TCTCTTTAGG CATTGGTTTC GAAGCGGGCA ACAAGCCGAA AGAACTGTAC
      ACGACGACAG CCGAAATTGG AGAGAAATCC GTAACCAAAG CTTCGCCCGT TGTTCGGCTT TCTTGACATG

2101  AGCGAAGAGG CAGTCAACGG GGAAACTCAG CAAGCGCACT TACAGGCGAT TAAAGAGCTG ATAGCGCGTG
      TCGCTTCTCC GTCAGTTGCC CCTTTGAGTC GTTCGCGTGA ATGTCCGCTA ATTTCTCGAC TATCGCGCAC

2171  ACAAAAACCA CCCAAGCGTG GTGATGTGGA GTATTGCCAA CGAACCGGAT ACCCGTCCGC AAGGTGCACG
      TGTTTTTGGT GGGTTCGCAC CACTACACCT CATAACGGTT GCTTGGCCTA TGGGCAGGCG TTCCACGTGC

2241  GGAATATTTC GCGCCACTGG CGGAAGCAAC GCGTAAACTC GACCCGACGC GTCCGATCAC CTGCGTCAAT
      CCTTATAAAG CGCGGTGACC GCGTTCGTTG CGCATTTGAG CTGGGCTGCG CAGGCTAGTG GACGCAGTTA

2311  GTAATGTTCT GCGACGCTCA CACCGATACC ATCAGCGATC TCTTTGATGT GCTGTGCCTG AACCGTTATT
      CATTACAAGA CGCTGCGAGT GTGGCTATGG TAGTCGCTAG AGAAACTACA CGACACGGAC TTGGCAATAA
```

FIG. 19 (FIG. $A_{2\ cont.}$)

```
2381  ACGGATGGTA TGTCCAAAGC GGCGATTTGG AAACGGCAGA GAAGGTACTG GAAAAAGAAC TTCTGGCCTG
      TGCCTACCAT ACAGGTTTCG CCGCTAAACC TTTGCCGTCT CTTCCATGAC CTTTTTCTTG AAGACCGGAC

2451  GCAGGAGAAA CTGCATCAGC CGATTATCAT CACCGAATAC GGCGTGGATA CGTTAGCCGG GCTGCACTCA
      CGTCCTCTTT GACGTAGTCG GCTAATAGTA GTGGCTTATG CCGCACCTAT GCAATCGGCC CGACGTGAGT

2521  ATGTACACCG ACATGTGGAG TGAAGAGTAT CAGTGTGCAT GGCTGGATAT GTATCACCGC GTCTTTGATC
      TACATGTGGC TGTACACCTC ACTTCTCATA GTCACACGTA CCGACCTATA CATAGTGGCG CAGAAACTAG

2591  GCGTCAGCGC CGTCGTCGGT GAACAGGTAT GGAATTTCGC CGATTTTGCG ACCTCGCAAG GCATATTGCG
      CGCAGTCGCG GCAGCAGCCA CTTGTCCATA CCTTAAAGCG GCTAAAACGC TGGAGCGTTC CGTATAACGC

2661  CGTTGGCGGT AACAAGAAAG GGATCTTCAC TCGCGACCGC AAACCGAAGT CGGCGGCTTT TCTGCTGCAA
      GCAACCGCCA TTGTTCTTTC CCTAGAAGTG AGCGCTGGCG TTTGGCTTCA GCCGCCGAAA AGACGACGTT

2731  AAACGCTGGA CTGGCATGAA CTTCGGTGAA AAACCGCAGC AGGGAGGCAA ACAATGAGAG CTCGGTTGTT
      TTTGCGACCT GACCGTACTT GAAGCCACTT TTTGGCGTCG TCCCTCCGTT TGTTACTCTC GAGCCAACAA
```

FIG. 20 (FIG. $A_{2\ cont.}$)

2801 GATGGATCTG TGATGCATGC AATAGCTGAT AATAGAACTT ACGCAAATAT TAGCAAAAAT ATATTAGACA
CTACCTAGAC ACTACGTACG TTATCGACTA TTATCTTGAA TGCGTTTATA ATCGTTTTTA TATAATCTGT

2871 ATACTACAAT TAACGATGAG TGTAGATGCT GTTATTTTGA ACCACAGATT AGGATTCTTG ATAGAGATGA
TATGATGTTA ATTGCTACTC ACATCTACGA CAATAAAACT TGGTGTCTAA TCCTAAGAAC TATCTCTACT

2941 GATGCTCAAT GGATCATCGT GTGATATGAA CAGACATTGT ATTATGATGA ATTTACCTGA TGTAGGCGAA
CTACGAGTTA CCTAGTAGCA CACTATACTT GTCTGTAACA TAATACTACT TAAATGGACT ACATCCGCTT

3011 TTTGGATCTA GTATGTTGGG GAAATATGAA CCTGACATGA TTAAGATTGC TCTTTCGGTG GCTGGCGGCC
AAACCTAGAT CATACAACCC CTTTATACTT GGACTGTACT AATTCTAACG AGAAAGCCAC CGACCGCCGG

3081 CGCTCGAGTA AAAAATGAAA AAATATTCTA ATTTATAGGA CGGTTTTGAT TTTCTTTTTT TCTATGCTAT
GCGAGCTCAT TTTTTACTTT TTTATAAGAT TAAATATCCT GCCAAAAACTA AAAGAAAAAA AGATACGATA

3151 AAATAATAAA TAGCGGCCGC ACCATGAAAG TGAAGGGGAT CAGGAAGAAT TATCAGCACT TGTGGAAATG
TTTATTATTT ATCGCCGGCG TGGTACTTTC ACTTCCCCTA GTCCTTCTTA ATAGTCGTGA ACACCTTTAC

FIG. 21 (FIG. $A_{2\ cont.}$)

```
3221  GGGCATCATG CTCCTTGGGA TGTTGATGAT CTGTAGTGCT GTAGAAAATT TGTGGGTCAC AGTTTATTAT
      CCCGTAGTAC GAGGAACCCT ACAACTACTA GACATCACGA CATCTTTTAA ACACCCAGTG TCAAATAATA

3291  GGGGTACCTG TGTGGAAAGA AGCAACCACC ACTCTATTTT GTGCATCAGA TGCTAAAGCA TATGATACAG
      CCCCATGGAC ACACCTTTCT TCGTTGGTGG TGAGATAAAA CACGTAGTCT ACGATTTCGT ATACTATGTC

3361  AGGTACATAA TGTTTGGGCC ACACATGCCT GTGTACCCAC AGACCCCAAC CCACAAGAAG TAGTATTGGA
      TCCATGTATT ACAAACCCGG TGTGTACGGA CACATGGGTG TCTGGGGTTG GGTGTTCTTC ATCATAACCT

3431  AAATGTGACA GAAAATTTTA ACATGTGGAA AAATAACATG GTAGAACAGA TGCATGAGGA TATAATCAGT
      TTTACACTGT CTTTTAAAAT TGTACACCTT TTTATTGTAC CATCTTGTCT ACGTACTCCT ATATTAGTCA

3501  TTATGGGATC AAAGCCTAAA GCCATGTGTA AAATTAACCC CACTCTGTGT TACTTTAAAT TGCACTGATT
      AATACCCTAG TTTCGGATTT CGGTACACAT TTTAATTGGG GTGAGACACA ATGAAATTTA ACGTGACTAA

3571  TGAGGAATGT TACTAATATC AATAATAGTA GTGAGGGAAT GAGAGGAGAA ATAAAAAACT GCTCTTTCAA
      ACTCCTTACA ATGATTATAG TTATTATCAT CACTCCCTTA CTCTCCTCTT TATTTTTTGA CGAGAAAGTT
```

FIG. 22 (FIG. $A_{2\ cont.}$)

```
3641 TATCACCACA AGCATAAGAG ATAAGGTGAA GAAAGACTAT GCACTTTTCT ATAGACTTGA TGTAGTACCA
     ATAGTGGTGT TCGTATTCTC TATTCCACTT CTTTCTGATA CGTGAAAAGA TATCTGAACT ACATCATGGT

3711 ATAGATAATG ATAATACTAG CTATAGGTTG ATAAATTGTA ATACCTCAAC CATTACACAG GCCTGTCCAA
     TATCTATTAC TATTATGATC GATATCCAAC TATTTAACAT TATGGAGTTG GTAATGTGTC CGGACAGGTT

3781 AGGTATCCTT TGAGCCAATT CCCATACATT ATTGTACCCC GGCTGGTTTT GCGATTCTAA AGTGTAAAGA
     TCCATAGGAA ACTCGGTTAA GGGTATGTAA TAACATGGGG CCGACCAAAA CGCTAAGATT TCACATTTCT

3851 CAAGAAGTTC AATGGAACAG GGCCATGTAA AAATGTCAGC ACAGTACAAT GTACACATGG AATTAGGCCA
     GTTCTTCAAG TTACCTTGTC CCGGTACATT TTTACAGTCG TGTCATGTTA CATGTGTACC TTAATCCGGT

3921 GTAGTGTCAA CTCAACTGCT GTTAAATGGC AGTCTAGCAG AAGAAGAGGT AGTAATTAGA TCTAGTAATT
     CATCACAGTT GAGTTGACGA CAATTTACCG TCAGATCGTC TTCTTCTCCA TCATTAATCT AGATCATTAA

3991 TCACAGACAA TGCAAAAAAC ATAATAGTAC AGTTGAAAGA ATCTGTAGAA ATTAATTGTA CAAGACCCAA
     AGTGTCTGTT ACGTTTTTTG TATTATCATG TCAACTTTCT TAGACATCTT TAATTAACAT GTTCTGGGTT
```

FIG. 23 (FIG. $A_{2\ cont.}$)

```
4061 CAACAATACA AGGAAAAGTA TACATATAGG ACCAGGAAGA GCATTTTATA CAACAGGAGA AATAATAGGA
     GTTGTTATGT TCCTTTTCAT ATGTATATCC TGGTCCTTCT CGTAAAATAT GTTGTCCTCT TTATTATCCT

4131 GATATAAGAC AAGCACATTG CAACATTAGT AGAACAAAAT GGAATAACAC TTTAAATCAA ATAGCTACAA
     CTATATTCTG TTCGTGTAAC GTTGTAATCA TCTTGTTTTA CCTTATTGTG AAATTTAGTT TATCGATGTT

4201 AATTAAAAGA ACAATTTGGG AATAATAAAA CAATAGTCTT TAATCAATCC TCAGGAGGGG ACCCAGAAAT
     TTAATTTTCT TGTTAAACCC TTATTATTTT GTTATCAGAA ATTAGTTAGG AGTCCTCCCC TGGGTCTTTA

4271 TGTAATGCAC AGTTTTAATT GTGGAGGGGA ATTCTTCTAC TGTAATTCAA CACAACTGTT TAATAGTACT
     ACATTACGTG TCAAAATTAA CACCTCCCCT TAAGAAGATG ACATTAAGTT GTGTTGACAA ATTATCATGA

4341 TGGAATTTTA ATGGTACTTG GAATTTAACA CAATCGAATG GTACTGAAGG AAATGACACT ATCACACTCC
     ACCTTAAAAT TACCATGAAC CTTAAATTGT GTTAGCTTAC CATGACTTCC TTTACTGTGA TAGTGTGAGG

4411 CATGTAGAAT AAACAAATT ATAAATATGT GGCAGGAAGT AGGAAAAGCA ATGTATGCCC CTCCCATCAG
     GTACATCTTA TTTGTTTAA TATTTATACA CCGTCCTTCA TCCTTTTCGT TACATACGGG GAGGGTAGTC
```

FIG. 24 (FIG. $A_{2\ cont.}$)

```
4481  AGGACAAATT AGATGCTCAT CAAATATTAC AGGGCTAATA TTAACAAGAG ATGGTGGAAC TAACAGTAGT
      TCCTGTTTAA TCTACGAGTA GTTTATAATG TCCCGATTAT AATTGTTCTC TACCACCTTG ATTGTCATCA

4551  GGGTCCGAGA TCTTCAGACC TGGGGGAGGA GATATGAGGG ACAATTGGAG AAGTGAATTA TATAAATATA
      CCCAGGCTCT AGAAGTCTGG ACCCCCTCCT CTATACTCCC TGTTAACCTC TTCACTTAAT ATATTTATAT

4621  AAGTAGTAAA AATTGAACCA TTAGGAGTAG CACCCACCAA GGCAAAAAGA AGAGTGGTGC AGAGAGAAAA
      TTCATCATTT TTAACTTGGT AATCCTCATC GTGGGTGGTT CCGTTTTTCT TCTCACCACG TCTCTCTTTT

4691  AAGAGCAGTG GGAACGATAG GAGCTATGTT CCTTGGGTTC TTGGGAGCAG CAGGAAGCAC TATGGGCGCA
      TTCTCGTCAC CCTTGCTATC CTCGATACAA GGAACCCAAG AACCCTCGTC GTCCTTCGTG ATACCCGCGT

4761  GCGTCAATAA CGCTGACGGT ACAGGCCAGA CTATTATTGT CTGGTATAGT GCAACAGCAG AACAATTTGC
      CGCAGTTATT GCGACTGCCA TGTCCGGTCT GATAATAACA GACCATATCA CGTTGTCGTC TTGTTAAACG

4831  TGAGGGCTAT TGAGGCGCAA CAGCATCTGT TGCAACTCAC AGTCTGGGGC ATCAAGCAGC TCCAGGCAAG
      ACTCCCGATA ACTCCGCGTT GTCGTAGACA ACGTTGAGTG TCAGACCCCG TAGTTCGTCG AGGTCCGTTC
```

FIG. 25 (FIG. $A_{2\ cont.}$)

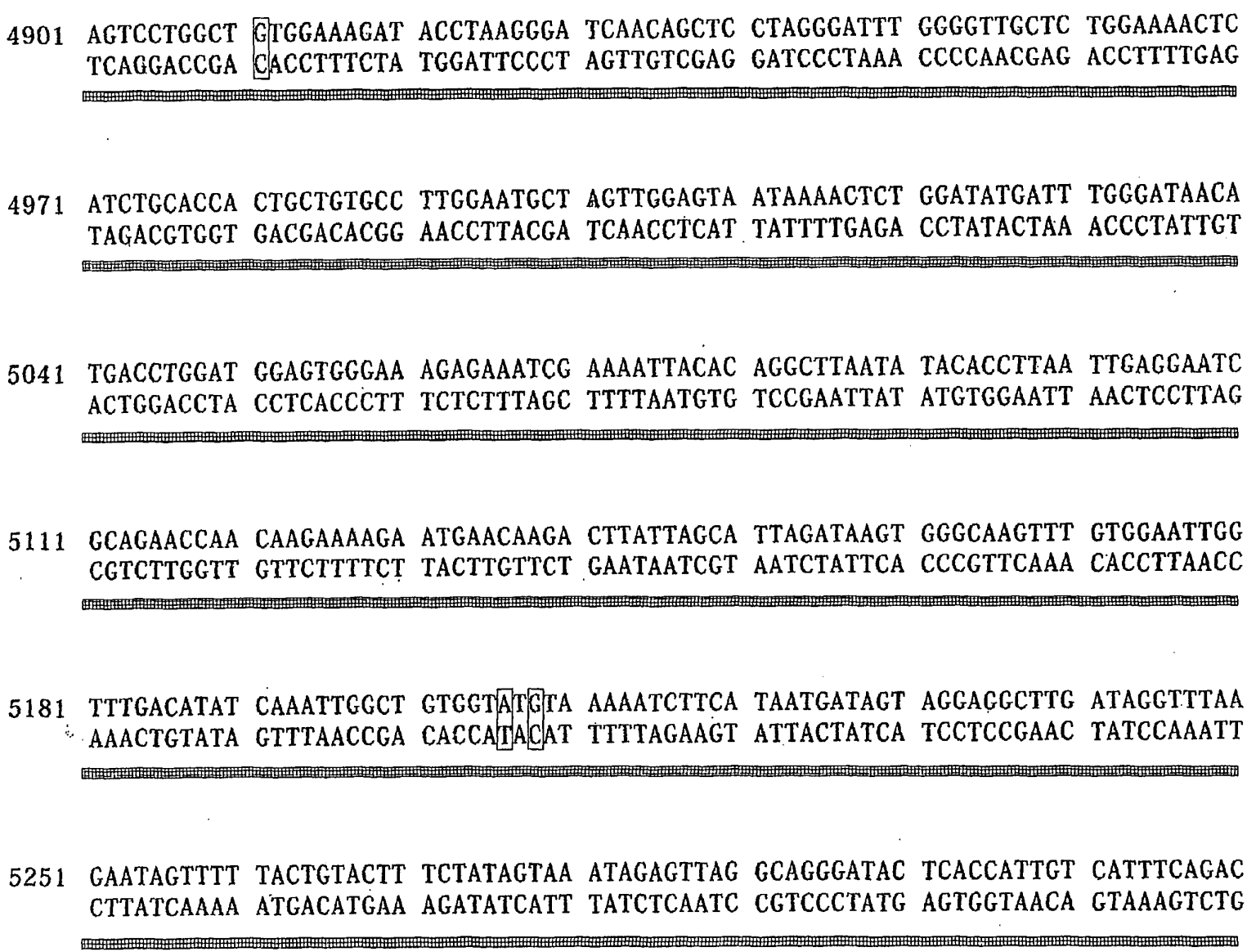

FIG. 26 (FIG. $A_{2\ cont.}$)

```
5321  CCACCTCCCA GCCCCGAGGG GACCCGACAG GCCCGAAGGA ATCGAAGAAG AAGGTGGAGA CAGAGACTAA
      GGTGGAGGGT CGGGGCTCCC CTGGGCTGTC CGGGCTTCCT TAGCTTCTTC TTCCACCTCT GTCTCTGATT

5391  TTTTTATGCG GCCGCTGGTA CCCAACCTAA AAATTGAAAA TAAATACAAA GGTTCTTGAG GGTTGTGTTA
      AAAAATACGC CGGCGACCAT GGGTTGGATT TTTAACTTTT ATTTATGTTT CCAAGAACTC CCAACACAAT

5461  AATTGAAAGC GAGAAATAAT CATAAATAAG CCCGGGGATC CTCTAGAGTC GACACCATGG GTGCGAGAGC
      TTAACTTTCG CTCTTTATTA GTATTTATTC GGGCCCCTAG GAGATCTCAG CTGTGGTACC CACGCTCTCG

5531  GTCAGTATTA AGCGGGGGAG AATTAGATCG ATGGGAAAAA ATTCGGTTAA GGCCAGGGGG AAAGAAAAAA
      CAGTCATAAT TCGCCCCCTC TTAATCTAGC TACCCTTTTT TAAGCCAATT CCGGTCCCCC TTTCTTTTTT

5601  TATAAATTAA AACATATAGT ATGGGCAAGC AGGGAGCTAG AACGATTCGC AGTTAATCCT GGCCTGTTAG
      ATATTTAATT TTGTATATCA TACCCGTTCG TCCCTCGATC TTGCTAAGCG TCAATTAGGA CCGGACAATC

5671  AAACATCAGA AGGCTGTAGA CAAATACTGG GACAGCTACA ACCATCCCTT CAGACAGGAT CAGAAGAACT
      TTTGTAGTCT TCCGACATCT GTTTATGACC CTGTCGATGT TGGTAGGGAA GTCTGTCCTA GTCTTCTTGA
```

FIG. 27 (FIG. $A_{2\ cont.}$)

```
5741  TAGATCATTA TATAATACAG TAGCAACCCT CTATTGTGTG CATCAAAGGA TAGAGATAAA AGACACCAAG
      ATCTAGTAAT ATATTATGTC ATCGTTGGGA GATAACACAC GTAGTTTCCT ATCTCTATTT TCTGTGGTTC

5811  GAAGCTTTAG ACAAGATAGA GGAAGAGCAA AACAAAAGTA AGAAAAAAGC ACAGCAAGCA GCAGCTGACA
      CTTCGAAATC TGTTCTATCT CCTTCTCGTT TTGTTTTCAT TCTTTTTTCG TGTCGTTCGT CGTCGACTGT

5881  CAGGACACAG CAATCAGGTC AGCCAAAATT ACCCTATAGT GCAGAACATC CAGGGGCAAA TGGTACATCA
      GTCCTGTGTC GTTAGTCCAG TCGGTTTTAA TGGGATATCA CGTCTTGTAG GTCCCCGTTT ACCATGTAGT

5951  GGCCATATCA CCTAGAACTT TAAATGCATG GGTAAAAGTA GTAGAAGAGA AGGCTTTCAG CCCAGAAGTG
      CCGGTATAGT GGATCTTGAA ATTTACGTAC CCATTTTCAT CATCTTCTCT TCCGAAAGTC GGGTCTTCAC

6021  ATACCCATGT TTTCAGCATT ATCAGAAGGA GCCACCCCAC AAGATTTAAA CACCATGCTA AACACAGTGG
      TATGGGTACA AAAGTCGTAA TAGTCTTCCT CGGTGGGGTG TTCTAAATTT GTGGTACGAT TTGTGTCACC

6091  GGGGACATCA AGCAGCCATG CAAATGTTAA AAGAGACCAT CAATGAGGAA GCTGCAGAAT GGGATAGAGT
      CCCCTGTAGT TCGTCGGTAC GTTTACAATT TTCTCTGGTA GTTACTCCTT CGACGTCTTA CCCTATCTCA
```

FIG. 28 (FIG. $A_{2\ cont.}$)

```
6161  GCATCCAGTG CATGCAGGGC CTATTGCACC AGGCCAGATG AGAGAACCAA GGGGAAGTGA CATAGCAGGA
      CGTAGGTCAC GTACGTCCCG GATAACGTGG TCCGGTCTAC TCTCTTGGTT CCCCTTCACT GTATCGTCCT

6231  ACTACTAGTA CCCTTCAGGA ACAAATAGGA TGGATGACAA ATAATCCACC TATCCCAGTA GGAGAAATTT
      TGATGATCAT GGGAAGTCCT TGTTTATCCT ACCTACTGTT TATTAGGTGG ATAGGGTCAT CCTCTTTAAA

6301  ATAAAAGATG GATAATCCTG GGATTAAATA AAATAGTAAG AATGTATAGC CCTACCAGCA TTCTGGACAT
      TATTTTCTAC CTATTAGGAC CCTAATTTAT TTTATCATTC TTACATATCG GGATGGTCGT AAGACCTGTA

6371  AAGACAAGGA CCAAAAGAAC CCTTTAGAGA CTATGTAGAC CGGTTCTATA AAACTCTAAG AGCCGAGCAA
      TTCTGTTCCT GGTTTTCTTG GGAAATCTCT GATACATCTG GCCAAGATAT TTTGAGATTC TCGGCTCGTT

6441  GCTTCACAGG AGGTAAAAAA TTGGATGACA GAAACCTTGT TGGTCCAAAA TGCGAACCCA GATTGTAAGA
      CGAAGTGTCC TCCATTTTTT AACCTACTGT CTTTGGAACA ACCAGGTTTT ACGCTTGGGT CTAACATTCT

6511  CTATTTTAAA AGCATTGGGA CCAGCGGCTA CACTAGAAGA AATGATGACA GCATGTCAGG GAGTAGGAGG
      GATAAAATTT TCGTAACCCT GGTCGCCGAT GTGATCTTCT TTACTACTGT CGTACAGTCC CTCATCCTCC
```

FIG. 29 (FIG. $A_{2\ cont.}$)

```
6581  ACCCGGCCAT AAGGCAAGAG TTTTGGCTGA AGCAATGAGC CAAGTAACAA ATTCAGCTAC CATAATGATG
      TGGGCCGGTA TTCCGTTCTC AAAACCGACT TCGTTACTCG GTTCATTGTT TAAGTCGATG GTATTACTAC

6651  CAGAGAGGCA ATTTTAGGAA CCAAAGAAAG ATTGTTAAGT GTTTCAATTC TGGCAAAGAA GGGCACACAG
      GTCTCTCCGT TAAAATCCTT GGTTTCTTTC TAACAATTCA CAAAGTTAAC ACCGTTTCTT CCCGTGTGTC

6721  CCAGAAATTG CAGGGCCCCT AGGAAAAAGG GCTGTTGGAA ATGTGGAAAG GAAGGACACC AAATGAAAGA
      GGTCTTTAAC GTCCCGGGGA TCCTTTTTCC CGACAACCTT TACACCTTTC CTTCCTGTGG TTTACTTTCT

6791  TTGTACTGAG AGACAGGCTA ATTTTTAGG GAAGATCTGG CCTTCCTACA AGGGAAGGCC AGGGAATTTT
      AACATGACTC TCTGTCCGAT TAAAAAATCC CTTCTAGACC GGAAGGATGT TCCCTTCCGG TCCCTTAAAA

6861  CTTCAGAGCA GACCAGAGCC AACAGCCCCA CCAGAAGAGA GCTTCAGGTC TGGGGTAGAG ACAACAACTC
      GAAGTCTCGT CTGGTCTCGG TTGTCGGGGT GGTCTTCTCT CGAAGTCCAG ACCCCATCTC TGTTGTTGAG

6931  CCCCTCAGAA GCAGGAGCCG ATAGACAAGG AACTGTATCC TTTAACTTCC CTCAGATCAC TCTTTGGCAA
      GGGGAGTCTT CGTCCTCGGC TATCTGTTCC TTGACATAGG AAATTGAAGG GAGTCTAGTG AGAAACCGTT
```

FIG. 30 (FIG. $A_{2\ cont.}$)

7001 CGACCCCTCG TCACAATAAA GATAGGGGGG CAACTAAAGG AAGCTCTATT AGATACAGGA GCAGATGATA
GCTGGGGAGC AGTGTTATTT CTATCCCCCC GTTGATTTCC TTCGAGATAA TCTATGTCCT CGTCTACTAT

7071 CAGTATTAGA AGAAATGAGT TTGCCAGGAA GATGGAAACC AAAAATGATA GGGGGAATTG GAGGTTTTAT
GTCATAATCT TCTTTACTCA AACGGTCCTT CTACCTTTGG TTTTTACTAT CCCCCTTAAC CTCCAAAATA

7141 CAAAGTAAGA CAGTATGATC AGATACTCAT AGAAATCTGT GGACATAAAG CTATAGGTAC AGTATTAGTA
GTTTCATTCT GTCATACTAG TCTATGAGTA TCTTTAGACA CCTGTATTTC GATATCCATG TCATAATCAT

7211 GGACCTACAC CTGTCAACAT AATTGGAAGA AATCTGTTGA CTCAGATTGG TTGCACTTTA AATTTTCCCA
CCTGGATGTG GACAGTTGTA TTAACCTTCT TTAGACAACT GAGTCTAACC AACGTGAAAT TTAAAAGGGT

7281 TTAGCCCTAT TGAGACTGTA CCAGTAAAAT TAAAGCCAGG AATGGATGGC CCAAAAGTTA AACAATGGCC
AATCGGGATA ACTCTGACAT GGTCATTTTA ATTTCGGTCC TTACCTACCG GGTTTTCAAT TTGTTACCGG

7351 ATTGACAGAA GAAAAAATAA AAGCATTAGT AGAAATTTGT ACAGAAATGG AAAAGGAAGG GAAAATTTCA
TAACTGTCTT CTTTTTTATT TTCGTAATCA TCTTTAAACA TGTCTTTACC TTTTCCTTCC CTTTTAAAGT

FIG. 31 (FIG. $A_{2\ cont.}$)

```
7421  AAAATTGGGC CTGAGAATCC ATACAATACT CCAGTATTTG CCATAAAGAA AAAAGACAGT ACTAAATGGA
      TTTTAACCCG GACTCTTAGG TATGTTATGA GGTCATAAAC GGTATTTCTT TTTTCTGTCA TGATTTACCT

7491  GGAAATTAGT AGATTTCAGA GAACTTAATA AGAGAACTCA AGACTTCTGG GAAGTTCAAT TAGGAATACC
      CCTTTAATCA TCTAAAGTCT CTTGAATTAT TCTCTTGAGT TCTGAAGACC CTTCAAGTTA ATCCTTATGG

7561  ACATCCCGCA GGGTTAAAAA AGAAAAAATC AGTAACAGTA CTGGATGTGG GTGATGCATA TTTTTCAGTT
      TGTAGGGCGT CCCAATTTTT TCTTTTTTAG TCATTGTCAT GACCTACACC CACTACGTAT AAAAAGTCAA

7631  CCCTTAGATG AAGACTTCAG GAAGTATACT GCATTTACCA TACCTAGTAT AAACAATGAG ACACCAGGGA
      GGGAATCTAC TTCTGAAGTC CTTCATATGA CGTAAATGGT ATGGATCATA TTTGTTACTC TGTGGTCCCT

7701  TTAGATATCA GTACAATGTG CTTCCACAGG GATGGAAAGG ATCACCAGCA ATATTCCAAA GTAGCATGAC
      AATCTATAGT CATGTTACAC GAAGGTGTCC CTACCTTTCC TAGTGGTCGT TATAAGGTTT CATCGTACTG

7771  AAAAATCTTA GAGCCTTTTA AAAAACAAAA TCCAGACATA GTTATCTATC AATACATGAA CGATTTGTAT
      TTTTTAGAAT CTCGGAAAAT TTTTTGTTTT AGGTCTGTAT CAATAGATAG TTATGTACTT GCTAAACATA
```

FIG. 32 (FIG. $A_{2\ cont.}$)

```
7841  GTAGGATCTG ACTTAGAAAT AGGGCAGCAT AGAACAAAAA TAGAGGAGCT GAGACAACAT CTGTTGAGGT
      CATCCTAGAC TGAATCTTTA TCCCGTCGTA TCTTGTTTTT ATCTCCTCGA CTCTGTTGTA GACAACTCCA

7911  GGGGACTTAC CACACCAGAC AAAAAACATC AGAAAGAACC TCCATTCCTT TGGATGGGTT ATGAACTCCA
      CCCCTGAATG GTGTGGTCTG TTTTTTGTAG TCTTTCTTGG AGGTAAGGAA ACCTACCCAA TACTTGAGGT

7981  TCCTGATAAA TGGACAGTAC AGCCTATAGT GCTGCCAGAA AAAGACAGCT GGACTGTCAA TGACATACAG
      AGGACTATTT ACCTGTCATG TCGGATATCA CGACGGTCTT TTTCTGTCGA CCTGACAGTT ACTGTATGTC

8051  AAGTTAGTGG GGAAATTGAA TACCGCAAGT CAGATTTACC CAGGGATTAA AGTAAGGCAA TTATGTAAAC
      TTCAATCACC CCTTTAACTT ATGGCGTTCA GTCTAAATGG GTCCCTAATT TCATTCCGTT AATACATTTG

8121  TCCTTAGAGG AACCAAAGCA CTAACAGAAG TAATACCACT AACAGAAGAA GCAGAGCTAG AACTGGCAGA
      AGGAATCTCC TTGGTTTCGT GATTGTCTTC ATTATGGTGA TTGTCTTCTT CGTCTCGATC TTGACCGTCT

8191  AAACAGAGAG ATTCTAAAAG AACCAGTACA TGGAGTGTAT TATGACCCAT CAAAAGACTT AATAGCAGAA
      TTTGTCTCTC TAAGATTTTC TTGGTCATGT ACCTCACATA ATACTGGGTA GTTTTCTGAA TTATCGTCTT
```

FIG. 33 (FIG. $A_{2\ cont.}$)

```
8261  ATACAGAAGC AGGGGCAAGG CCAATGGACA TATCAAATTT ATCAAGAGCC ATTTAAAAAT CTGAAAACAG
      TATGTCTTCG TCCCCGTTCC GGTTACCTGT ATAGTTTAAA TAGTTCTCGG TAAATTTTTA GACTTTTGTC

8331  GAAAATATGC AAGAATGAGG GGTGCCCACA CTAATGATGT AAAACAATTA ACAGAGGCAG TGCAAAAAAT
      CTTTTATACG TTCTTACTCC CCACGGGTGT GATTACTACA TTTTGTTAAT TGTCTCCGTC ACGTTTTTTA

8401  AACCACAGAA AGCATAGTAA TATGGGGAAA GACTCCTAAA TTTAAACTAC CCATACAAAA GGAAACATGG
      TTGGTGTCTT TCGTATCATT ATACCCCTTT CTGAGGATTT AAATTTGATG GGTATGTTTT CCTTTGTACC

8471  GAAACATGGT GGACAGAGTA TTGGCAAGCC ACCTGGATTC CTGAGTGGGA GTTTGTTAAT ACCCCTCCTT
      CTTTGTACCA CCTGTCTCAT AACCGTTCGG TGGACCTAAG GACTCACCCT CAAACAATTA TGGGGAGGAA

8541  TAGTGAAATT ATGGTACCAG TTAGAGAAAG AACCCATAGT AGGAGCAGAA ACCTTCTATG TAGATGGGGC
      ATCACTTTAA TACCATGGTC AATCTCTTTC TTGGGTATCA TCCTCGTCTT TGGAAGATAC ATCTACCCCG

8611  AGCTAACAGG GAGACTAAAT TAGGAAAAGC AGGATATGTT ACTAACAAAG GAAGACAAAA GGTTGTCCCC
      TCGATTGTCC CTCTGATTTA ATCCTTTTCG TCCTATACAA TGATTGTTTC CTTCTGTTTT CCAACAGGGG
```

FIG. 34 (FIG. $A_{2\ cont.}$)

```
8681  CTAACTAACA CAACAAATCA GAAAACTCAG TTACAAGCAA TTTATCTAGC TTTGCAGGAT TCAGGATTAG
      GATTGATTGT GTTGTTTAGT CTTTTGAGTC AATGTTCGTT AAATAGATCG AAACGTCCTA AGTCCTAATC

8751  AAGTAAACAT AGTAACAGAC TCACAATATG CATTAGGAAT CATTCAAGCA CAACCAGATA AAAGTGAATC
      TTCATTTGTA TCATTGTCTG AGTGTTATAC GTAATCCTTA GTAAGTTCGT GTTGGTCTAT TTTCACTTAG

8821  AGAGTTAGTC AATCAAATAA TAGAGCAGTT AATAAAAAAG GAAAAGGTCT ATCTGGCATG GGTACCAGCA
      TCTCAATCAG TTAGTTTATT ATCTCGTCAA TTATTTTTTC CTTTTCCAGA TAGACCGTAC CCATGGTCGT

8891  CACAAAGGAA TTGGAGGAAA TGAACAAGTA GATAAATTAG TCAGTGCTGG AATCAGGAAA ATACTATTTT
      GTGTTTCCTT AACCTCCTTT ACTTGTTCAT CTATTTAATC AGTCACGACC TTAGTCCTTT TATGATAAAA

8961  TAGATGGAAT AGATAAGGCC CAAGATGAAC ATTAGTTTTT ATGTCGACCT GCAGGGAAAG TTTTATAGGT
      ATCTACCTTA TCTATTCCGG GTTCTACTTG TAATCAAAAA TACAGCTGGA CGTCCCTTTC AAAATATCCA

9031  AGTTGATAGA ACAAAATACA TAATTTTGTA AAAATAAATC ACTTTTTATA CTAATATGAC ACGATTACCA
      TCAACTATCT TGTTTTATGT ATTAAAACAT TTTTATTTAG TGAAAAATAT GATTATACTG TGCTAATGGT
```

FIG. 35 (FIG. $A_{2\ cont.}$)

```
9101  ATACTTTTGT TACTAATATC ATTAGTATAC GCTACACCTT TTCCTCAGAC ATCTAAAAAA ATAGGTGATG
      TATGAAAACA ATGATTATAG TAATCATATG CGATGTGGAA AAGGAGTCTG TAGATTTTTT TATCCACTAC

9171  ATGCAACTTT ATCATGTAAT CGAAATAATA CAAATGACTA CGTTGTTATG AGTGCTTGGT ATAAGGAGCC
      TACGTTGAAA TAGTACATTA GCTTTATTAT GTTTACTGAT GCAACAATAC TCACGAACCA TATTCCTCGG

9241  CAATTCCATT ATTCTTTTAG CTGCTAAAAG CGACGTCTTG TATTTTGATA ATTATACCAA GGATAAAATA
      GTTAAGGTAA TAAGAAAATC GACGATTTTC GCTGCAGAAC ATAAAACTAT TAATATGGTT CCTATTTTAT

9311  TCTTACGACT CTCCATACGA TGATCTAGTT ACAACTATCA CAATTAAATC ATTGACTGCT AGAGATGCCG
      AGAATGCTGA GAGGTATGCT ACTAGATCAA TGTTGATAGT GTTAATTTAG TAACTGACGA TCTCTACGGC

9381  GTACTTATGT ATGTGCATTC TTTATGACAT CGCCTACAAA TGACACTGAT AAAGTAGATT ATGAAGAATA
      CATGAATACA TACACGTAAG AAATACTGTA GCGGATGTTT ACTGTGACTA TTTCATCTAA TACTTCTTAT

9451  CTCCACAGAG TTGATTGTAA ATACAGATAG TGAATCGACT ATAGACATAA TACTATCTGG ATCTACACAT
      GAGGTGTCTC AACTAACATT TATGTCTATC ACTTAGCTGA TATCTGTATT ATGATAGACC TAGATGTGTA
```

FIG. 36 (FIG. $A_{2\ cont.}$)

```
9521  TCACCAGAAA CTAGTTAAGC TTGTCTCCCT ATAGTGAGTC GTATTAGAGC TTGGCGTAAT CATGGTCATA
      AGTGGTCTTT GATCAATTCG AACAGAGGGA TATCACTCAG CATAATCTCG AACCGCATTA GTACCAGTAT

9591  GCTGTTTCCT GTGTGAAATT GTTATCCGCT CACAATTCCA CACAACATAC GAGCCGGAAG CATAAAGTGT
      CGACAAAGGA CACACTTTAA CAATAGGCGA GTGTTAAGGT GTGTTGTATG CTCGGCCTTC GTATTTCACA

9661  AAAGCCTGGG GTGCCTAATG AGTGAGCTAA CTCACATTAA TTGCGTTGCG CTCACTGCCC GCTTTCGAGT
      TTTCGGACCC CACGGATTAC TCACTCGATT GAGTGTAATT AACGCAACGC GAGTGACGGG CGAAAGCTCA

9731  CGGGAAACCT GTCGTGCCAG CTGCATTAAT GAATCGGCCA ACGCGCGGGG AGAGGCGGTT TGCGTATTGG
      GCCCTTTGGA CAGCACGGTC GACGTAATTA CTTAGCCGGT TGCGCGCCCC TCTCCGCCAA ACGCATAACC

9801  GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC TGCGGCGAGC GGTATCAGCT
      CGCGAGAAGG CGAAGGAGCG AGTGACTGAG CGACGCGAGC CAGCAAGCCG ACGCCGCTCG CCATAGTCGA

9871  CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG ATAACGCAGG AAAGAACATG TGAGCAAAAG
      GTGAGTTTCC GCCATTATGC CAATAGGTGT CTTAGTCCCC TATTGCGTCC TTTCTTGTAC ACTCGTTTTC

9941  GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG CCGCGTTGCT GGCGTTTTTC GATAGGCTCC GCCCCCCTGA
      CGGTCGTTTT CCGGTCCTTG GCATTTTTCC GGCGCAACGA CCGCAAAAAG CTATCCGAGG CGGGGGGACT
```

FIG. 37 (FIG. $A_{2\ cont.}$)

```
10011  CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG
       GCTCGTAGTG TTTTTAGCTG CGAGTTCAGT CTCCACCGCT TTGGGCTGTC CTGATATTTC TATGGTCCGC

10081  TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT
       AAAGGGGGAC CTTCGAGGGA GCACGCGAGA GGACAAGGCT GGGACGGCGA ATGGCCTATG GACAGGCGGA

10151  TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC ATAGCTCACG CTGTAGGTAT CTCAGTTCGG TGTAGGTCGT
       AAGAGGGAAG CCCTTCGCAC CGCGAAAGAG TATCGAGTGC GACATCCATA GAGTCAAGCC ACATCCAGCA

10221  TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT GCGCCTTATC CGGTAACTAT
       AGCGAGGTTC GACCCGACAC ACGTGCTTGG GGGGCAAGTC GGGCTGGCGA CGCGGAATAG GCCATTGATA

10291  CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA
       GCAGAACTCA GGTTGGGCCA TTCTGTGCTG AATAGCGGTG ACCGTCGTCG GTGACCATTG TCCTAATCGT

10361  GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC
       CTCGCTCCAT ACATCCGCCA CGATGTCTCA AGAACTTCAC CACCGGATTG ATGCCGATGT GATCTTCCTG

10431  AGTATTTGGT ATCTGCGCTC TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC
       TCATAAACCA TAGACGCGAG ACGACTTCGG TCAATGGAAG CCTTTTTCTC AACCATCGAG AACTAGGCCG
```

FIG. 38 (FIG. $A_{2\ cont.}$)

```
10501  AAACAAACCA CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT
       TTTGTTTGGT GGCGACCATC GCCACCAAAA AAACAAACGT TCGTCGTCTA ATGCGCGTCT TTTTTTCCTA

10571  CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC GTTAAGGGAT
       GAGTTCTTCT AGGAAACTAG AAAAGATGCC CCAGACTGCG AGTCACCTTG CTTTTGAGTG CAATTCCCTA

10641  TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA
       AAACCAGTAC TCTAATAGTT TTTCCTAGAA GTGGATCTAG GAAAATTTAA TTTTTACTTC AAAATTTAGT

10711  ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT CAGTGAGGCA CCTATCTCAG
       TAGATTTCAT ATATACTCAT TTGAACCAGA CTGTCAATGG TTACGAATTA GTCACTCCGT GGATAGAGTC

10781  CGATCTGTCT ATTTCGTTCA TCCATAGTTG CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG
       GCTAGACAGA TAAAGCAAGT AGGTATCAAC GGACTGAGGG GCAGCACATC TATTGATGCT ATGCCCTCCC

10851  CTTACCATCT GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA TTTATCAGCA
       GAATGGTAGA CCGGGGTCAC GACGTTACTA TGGCGCTCTG GGTGCGAGTG GCCGAGGTCT AAATAGTCGT

10921  ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC CTGCAACTTT ATCCGCCTCC ATCCAGTCTA
       TATTTGGTCG GTCGGCCTTC CCGGCTCGCG TCTTCACCAG GACGTTGAAA TAGGCGGAGG TAGGTCAGAT
```

FIG. 39 (FIG. $A_{2\ cont.}$)

```
10991  TTAATTGTTG CCGGGAAGCT AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGGCATTGC
       AATTAACAAC GGCCCTTCGA TCTCATTCAT CAAGCGGTCA ATTATCAAAC GCGTTGCAAC AACCGTAACG

11061  TACAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT CCGGTTCCCA ACGATCAAGG
       ATGTCCGTAG CACCACAGTG CGAGCAGCAA ACCATACCGA AGTAAGTCGA GGCCAAGGGT TGCTAGTTCC

11131  CGAGTTACAT GATCCCCCAT GTTGTGCAAA AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA
       GCTCAATGTA CTAGGGGGTA CAACACGTTT TTTCGCCAAT CGAGGAAGCC AGGAGGCTAG CAACAGTCTT

11201  GTAAGTTGGC CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG TCATGCCATC
       CATTCAACCG GCGTCACAAT AGTGAGTACC AATACCGTCG TGACGTATTA AGAGAATGAC AGTACGGTAG

11271  CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG TCATTCTGAG AATAGTGTAT GCGGCGACCG
       GCATTCTACG AAAAGACACT GACCACTCAT GAGTTGGTTC AGTAAGACTC TTATCACATA CGCCGCTGGC

11341  AGTTGCTCTT GCCCGGCGTC AATACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA
       TCAACGAGAA CGGGCCGCAG TTATGCCCTA TTATGGCGCG GTGTATCGTC TTGAAATTTT CACGAGTAGT
```

FIG. 40 (FIG. $A_{2\ cont.}$)

```
11411  TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG AGATCCAGTT CGATGTAACC
       AACCTTTTGC AAGAAGCCCC GCTTTTGAGA GTTCCTAGAA TGGCGACAAC TCTAGGTCAA GCTACATTGG

11481  CACTCGTGCA CCCAACTGAT CTTCAGCATC TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA
       GTGAGCACGT GGGTTGACTA GAAGTCGTAG AAAATGAAAG TGGTCGCAAA GACCCACTCG TTTTTGTCCT

11551  AGGCAAAATG CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC TTCCTTTTTC
       TCCGTTTTAC GGCGTTTTTT CCCTTATTCC CGCTGTGCCT TTACAACTTA TGAGTATGAG AAGGAAAAAG

11621  AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG CGGATACATA TTTGAATGTA TTTAGAAAAA
       TTATAATAAC TTCGTAAATA GTCCCAATAA CAGAGTACTC GCCTATGTAT AAACTTACAT AAATCTTTTT

11691  TAAACAAATA GGGGTTCCGC GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC
       ATTTGTTTAT CCCCAAGGCG CGTGTAAAGG GGCTTTTCAC GGTGGACTGC AGATTCTTTG GTAATAATAG

11761  ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTCGC GCGTTTCGGT GATGACGGTG
       TACTGTAATT GGATATTTTT ATCCGCATAG TGCTCCGGGA AAGCAGAGCG CGCAAAGCCA CTACTGCCAC
```

FIG. 41 (FIG. $A_{2\ cont.}$)

```
11831  AAAACCTCTG ACACATGCAG CTCCCGGAGA CGGTCACAGC TTGTCTGTAA GCGGATGCCG GGAGCAGACA
       TTTTGGAGAC TGTGTACGTC GAGGGCCTCT GCCAGTGTCG AACAGACATT CGCCTACGGC CCTCGTCTGT

11901  AGCCCGTCAG GGCGCGTCAG CGGGTGTTGG CGGGTGTCGG GGCTGGCTTA ACTATGCCGC ATCAGAGCAG
       TCGGGCAGTC CCGCGCAGTC GCCCACAACC GCCCACAGCC CCGACCGAAT TGATACGCCG TAGTCTCGTC

11971  ATTGTACTGA GAGTGCACCA TATGCGGTGT GAAATACCGC ACAGATGCGT AAGGAGAAAA TACCGCATCA
       TAACATGACT CTCACGTGGT ATACGCCACA CTTTATGGCG TGTCTACGCA TTCCTCTTTT ATGGCGTAGT

12041  GGCGCCATTC GCCATTCAGG CTGCGCAACT GTTGGGAAGG GCGATCGGTG CGGGCCTCTT CGCTATTACG
       CCGCGGTAAG CGGTAAGTCC GACGCGTTGA CAACCCTTCC CGCTAGCCAC GCCCGGAGAA GCGATAATGC

12111  CCAGCTGGCG AAAGGGGGAT GTGCTGCAAG GCGATTAAGT TGGGTAACGC CAGGGTTTTC CCAGTCACGA
       GGTCGACCGC TTTCCCCCTA CACGACGTTC CGCTAATTCA ACCCATTGCG GTCCCAAAAG GGTCAGTGCT

12181  CGTTGTAAAA CGACGGCCAG TGAATTGGAT TTAGGTGACA CTATA
       GCAACATTTT GCTGCCGGTC ACTTAACCTA AATCCACTGT GATAT
```

FIG. 42 (FIG. $A_{2\ cont.}$)

Text File of pLW-48 and the Included Individual HIV Genes and Their Promoters

Entire pLW-48 plasmid sequence:

GAATTCGTTGGTGGTCGCCATGGATGGTGTTATTGTATACTGTCTAAACGCG
TTAGTAAAACATGGCGAGGAAATAAATCATATAAAAAATGATTTCATGATTAA
ACCATGTTGTGAAAAAGTCAAGAACGTTCACATTGGCGGACAATCTAAAAAC
AATACAGTGATTGCAGATTTGCCATATATGGATAATGCGGTATCCGATGTAT
GCAATTCACTGTATAAAAAGAATGTATCAAGAATATCCAGATTTGCTAATTTG
ATAAAGATAGATGACGATGACAAGACTCCTACTGGTGTATATAATTATTTTAA
ACCTAAAGATGCCATTCCTGTTATTATATCCATAGGAAAGGATAGAGATGTTT
GTGAACTATTAATCTCATCTGATAAAGCGTGTGCGTGTATAGAGTTAAATTCA
TATAAAGTAGCCATTCTTCCCATGGATGTTTCCTTTTTTTACCAAAGGAAATGC
ATCATTGATTATTCTCCTGTTTGATTTCTCTATCGATGCGGCACCTCTCTTAA
GAAGTGTAACCGATAATAATGTTATTATATCTAGACACCAGCGTCTACATGA
CGAGCTTCCGAGTTCCAATTGGTTCAAGTTTTACATAAGTATAAAGTCCGAC
TATTGTTCTATATTATATATGGTTGTTGATGGATCTGTGATGCATGCAATAGC
TGATAATAGAACTTACGCAAATATTAGCAAAAATATATTAGACAATACTACAA
TTAACGATGAGTGTAGATGCTGTTATTTTGAACCACAGATTAGGATTCTTGAT
AGAGATGAGATGCTCAATGGATCATCGTGTGATATGAACAGACATTGTATTA
TGATGAATTTACCTGATGTAGGCGAATTTGGATCTAGTATGTTGGGGAAATA
TGAACCTGACATGATTAAGATTGCTCTTTCGGTGGCTGGGTACCAGGCGCG
CCTTTCATTTTGTTTTTTTCTATGCTATAAATGGTACGTCCTGTAGAAACCCC
AACCCGTGAAATCAAAAAACTCGACGGCCTGTGGGCATTCAGTCTGGATCG
CGAAAACTGTGGAATTGATCAGCGTTGGTGGGAAAGCGCGTTACAAGAAAG
CCGGGCAATTGCTGTGCCAGGCAGTTTTAACGATCAGTTCGCCGATGCAGA
TATTCGTAATTATGCGGGCAACGTCTGGTATCAGCGCGAAGTCTTTATACCG
AAAGGTTGGGCAGGCCAGCGTATCGTGCTGCGTTTCGATGCGGTCACTCAT
TACGGCAAAGTGTGGGTCAATAATCAGGAAGTGATGGAGCATCAGGGCGG
CTATACGCCATTTGAAGCCGATGTCACGCCGTATGTTATTGCCGGGAAAAG
TGTACGTATCACCGTTTGTGTGAACAACGAACTGAACTGGCAGACTATCCC
GCCGGGAATGGTGATTACCGACGAAAACGGCAAGAAAAAGCAGTCTTACTT
CCATGATTTCTTTAACTATGCCGGAATCCATCGCAGCGTAATGCTCTACACC
ACGCCGAACACCTGGGTGGACGATATCACCGTGGTGACGCATGTCGCGCA
AGACTGTAACCACGCGTCTGTTGACTGGCAGGTGGTGGCCAATGGTGATGT
CAGCGTTGAACTGCGTGATGCGGATCAACAGGTGGTTGCAACTGGACAAG
GCACTAGCGGGACTTTGCAAGTGGTGAATCCGCACCTCTGGCAACCGGGT
GAAGGTTATCTCTATGAACTGTGCGTCACAGCCAAAAGCCAGACAGAGTGT
GATATCTACCCGCTTCGCGTCGGCATCCGGTCAGTGGCAGTGAAGGGCGA
ACAGTTCCTGATTAACCACAAACCGTTCTACTTTACTGGCTTTGGTCGTCAT
GAAGATGCGGACTTGCGTGGCAAAGGATTCGATAACGTGCTGATGGTGCAC
GACCACGCATTAATGGACTGGATTGGGGCCAACTCCTACCGTACCTCGCAT
TACCCTTACGCTGAAGAGATGCTCGACTGGGCAGATGAACATGGCATCGTG

FIG. 43

(Figure B_1)

GTGATTGATGAAACTGCTGCTGTCGGCTTTAACCTCTCTTTAGGCATTGGTT
TCGAAGCGGGCAACAAGCCGAAAGAACTGTACAGCGAAGAGGCAGTCAAC
GGGGAAACTCAGCAAGCGCACTTACAGGCGATTAAAGAGCTGATAGCGCGT
GACAAAAACCACCCAAGCGTGGTGATGTGGAGTATTGCCAACGAACCGGAT
ACCCGTCCGCAAGGTGCACGGGAATATTTCGCGCCACTGGCGGAAGCAAC
GCGTAAACTCGACCCGACGCGTCCGATCACCTGCGTCAATGTAATGTTCTG
CGACGCTCACACCGATACCATCAGCGATCTCTTTGATGTGCTGTGCCTGAA
CCGTTATTACGGATGGTATGTCCAAAGCGGCGATTTGGAAACGGCAGAGAA
GGTACTGGAAAAAGAACTTCTGGCCTGGCAGGAGAAACTGCATCAGCCGAT
TATCATCACCGAATACGGCGTGGATACGTTAGCCGGGCTGCACTCAATGTA
CACCGACATGTGGAGTGAAGAGTATCAGTGTGCATGGCTGGATATGTATCA
CCGCGTCTTTGATCGCGTCAGCGCCGTCGTCGGTGAACAGGTATGGAATTT
CGCCGATTTTGCGACCTCGCAAGGCATATTGCGCGTTGGCGGTAACAAGAA
AGGGATCTTCACTCGCGACCGCAAACCGAAGTCGGCGGCTTTTCTGCTGCA
AAAACGCTGGACTGGCATGAACTTCGGTGAAAAACCGCAGCAGGGAGGCA
AACAATGAGAGCTCGGTTGTTGATGGATCTGTGATGCATGCAATAGCTGATA
ATAGAACTTACGCAAATATTAGCAAAAATATATTAGACAATACTACAATTAAC
GATGAGTGTAGATGCTGTTATTTTGAACCACAGATTAGGATTCTTGATAGAG
ATGAGATGCTCAATGGATCATCGTGTGATATGAACAGACATTGTATTATGAT
GAATTTACCTGATGTAGGCGAATTTGGATCTAGTATGTTGGGGAAATATGAA
CCTGACATGATTAAGATTGCTCTTTCGGTGGCTGGCGGCCCGCTCGAGTAA
AAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTCTAT
GCTATAAATAATAAATAGCGGCCGCACCATGAAAGTGAAGGGGATCAGGAA
GAATTATCAGCACTTGTGGAAATGGGGCATCATGCTCCTTGGGATGTTGATG
ATCTGTAGTGCTGTAGAAAATTTGTGGGTCACAGTTTATTATGGGGTACCTG
TGTGGAAAGAAGCAACCACCACTCTATTTTGTGCATCAGATGCTAAAGCATA
TGATACAGAGGTACATAATGTTTGGGCCACACATGCCTGTGTACCCACAGA
CCCCAACCCACAAGAAGTAGTATTGGAAAATGTGACAGAAAATTTTAACATG
TGGAAAAATAACATGGTAGAACAGATGCATGAGGATATAATCAGTTTATGGG
ATCAAAGCCTAAAGCCATGTGTAAAATTAACCCCACTCTGTGTTACTTTAAAT
TGCACTGATTTGAGGAATGTTACTAATATCAATAATAGTAGTGAGGGAATGA
GAGGAGAAATAAAAAACTGCTCTTTCAATATCACCACAAGCATAAGAGATAA
GGTGAAGAAAGACTATGCACTTTTcTATAGACTTGATGTAGTACCAATAGATA
ATGATAATACTAGCTATAGGTTGATAAATTGTAATACCTCAACCATTACACAG
GCCTGTCCAAAGGTATCCTTTGAGCCAATTCCCATACATTATTGTACCCCGG
CTGGTTTTGCGATTCTAAAGTGTAAAGACAAGAAGTTCAATGGAACAGGGCC
ATGTAAAAATGTCAGCACAGTACAATGTACACATGGAATTAGGCCAGTAGTG
TCAACTCAACTGCTGTTAAATGGCAGTCTAGCAGAAGAAGAGGTAGTAATTA
GATCTAGTAATTTCACAGACAATGCAAAAAACATAATAGTACAGTTGAAAGAA
TCTGTAGAAATTAATTGTACAAGACCCAACAACAATACAAGGAAAAGTATAC
ATATAGGACCAGGAAGAGCATTTTATACAACAGGAGAAATAATAGGAGATAT
AAGACAAGCACATTGCAACATTAGTAGAACAAAATGGAATAACACTTTAAAT
CAAATAGCTACAAAATTAAAAGAACAATTTGGGAATAATAAAACAATAGTCTT
TAATCAATCCTCAGGAGGGGACCCAGAAATTGTAATGCACAGTTTTAATTGT
GGAGGGGAATTCTTCTACTGTAATTCAACACAACTGTTTAATAGTACTTGGA
ATTTTAATGGTACTTGGAATTTAACACAATCGAATGGTACTGAAGGAAATGA

FIG. 44

(Figure B_2)

CACTATCACACTCCCATGTAGAATAAAACAAATTATAAATATGTGGCAGGAA
GTAGGAAAAGCAATGTATGCCCCTCCCATCAGAGGACAAATTAGATGCTCAT
CAAATATTACAGGGCTAATATTAACAAGAGATGGTGGAACTAACAGTAGTGG
GTCCGAGATCTTCAGACCTGGGGGAGGAGATATGAGGGACAATTGGAGAA
GTGAATTATATAAATATAAAGTAGTAAAAATTGAACCATTAGGAGTAGCACCC
ACCAAGGCAAAAAGAAGAGTGGTGCAGAGAGAAAAAAGAGCAGTGGGAAC
GATAGGAGCTATGTTCCTTGGGTTCTTGGGAGCAGCAGGAAGCACTATGGG
CGCAGCGTCAATAACGCTGACGGTACAGGCCAGACTATTATTGTCTGGTAT
AGTGCAACAGCAGAACAATTTGCTGAGGGCTATTGAGGCGCAACAGCATCT
GTTGCAACTCACAGTCTGGGGCATCAAGCAGCTCCAGGCAAGAGTCCTGG
CTGTGGAAAGATACCTAAGGGATCAACAGCTCCTAGGGATTTGGGGTTGCT
CTGGAAAACTCATCTGCACCACTGCTGTGCCTTGGAATGCTAGTTGGAGTA
ATAAAACTCTGGATATGATTTGGGATAACATGACCTGGATGGAGTGGGAAA
GAGAAATCGAAAATTACACAGGCTTAATATACACCTTAATTGAGGAATCGCA
GAACCAACAAGAAAAGAATGAACAAGACTTATTAGCATTAGATAAGTGGGCA
AGTTTGTGGAATTGGTTTGACATATCAAATTGGCTGTGGTATGTAAAAATCTT
CATAATGATAGTAGGAGGCTTGATAGGTTTAAGAATAGTTTTTACTGTACTTT
CTATAGTAAATAGAGTTAGGCAGGGATACTCACCATTGTCATTTCAGACCCA
CCTCCCAGCCCCGAGGGGACCCGACAGGCCCGAAGGAATCGAAGAAGAAG
GTGGAGACAGAGACTAATTTTTATGCGGCCGCTGGTACCCAACCTAAAAATT
GAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGCGAGAAAT
AATCATAAATAAGCCCGGGGATCCTCTAGAGTCGACACCATGGGTGCGAGA
GCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGAAAAAATTCGGTTA
AGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTATGGGCAAGCA
GGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAG
GCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAG
AAGAACTTAGATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAA
AGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAG
CAAAACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGCTGACACAGGACAC
AGCAATCAGGTCAGCCAAAATTACCCTATAGTGCAGAACATCCAGGGGCAA
ATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCATGGGTAAAAGTAG
TAGAAGAGAAGGCTTTCAGCCCAGAAGTGATACCCATGTTTTCAGCATTATC
AGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAAACACAGTGGGGGGG
ACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGGAAGCTGC
AGAATGGGATAGAGTGCATCCAGTGCATGCAGGGCCTATTGCACCAGGCCA
GATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACTACTAGTACCCTTCA
GGAACAAATAGGATGGATGACAAATAATCCACCTATCCCAGTAGGAGAAATT
TATAAAAGATGGATAATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCC
TACCAGCATTCTGGACATAAGACAAGGACCAAAAGAACCCTTTAGAGACTAT
GTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAAGCTTCACAGGAGGTA
AAAAATTGGATGACAGAAACCTTGTTGGTCCAAAATGCGAACCCAGATTGTA
AGACTATTTTAAAAGCATTGGGACCAGCGGCTACACTAGAAGAAATGATGAC
AGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTG
AAGCAATGAGCCAAGTAACAAATTCAGCTACCATAATGATGCAGAGAGGCA
ATTTTAGGAACCAAAGAAAGATTGTTAAGTGTTTCAATTGTGGCAAAGAAGG
GCACACAGCCAGAAATTGCAGGGCCCCTAGGAAAAAGGGCTGTTGGAAAT

FIG. 45

(Figure B_3)

GTGGAAAGGAAGGACACCAAATGAAAGATTGTACTGAGAGACAGGCTAATT
TTTTAGGGAAGATCTGGCCTTCCTACAAGGGAAGGCCAGGGAATTTTCTTCA
GAGCAGACCAGAGCCAACAGCCCCACCAGAAGAGAGCTTCAGGTCTGGGG
TAGAGACAACAACTCCCCCTCAGAAGCAGGAGCCGATAGACAAGGAACTGT
ATCCTTTAACTTCCCTCAGATCACTCTTTGGCAACGACCCCTCGTCACAATA
AAGATAGGGGGGCAACTAAAGGAAGCTCTATTAGATACAGGAGCAGATGAT
ACAGTATTAGAAGAAATGAGTTTGCCAGGAAGATGGAAACCAAAAATGATAG
GGGGAATTGGAGGTTTTATCAAAGTAAGACAGTATGATCAGATACTCATAGA
AATCTGTGGACATAAAGCTATAGGTACAGTATTAGTAGGACCTACACCTGTC
AACATAATTGGAAGAAATCTGTTGACTCAGATTGGTTGCACTTTAAATTTTCC
CATTAGCCCTATTGAGACTGTACCAGTAAAATTAAAGCCAGGAATGGATGGC
CCAAAAGTTAAACAATGGCCATTGACAGAAGAAAAAATAAAAGCATTAGTAG
AAATTTGTACAGAAATGGAAAAGGAAGGGAAAATTTCAAAAATTGGGCCTGA
GAATCCATACAATACTCCAGTATTTGCCATAAAGAAAAAAGACAGTACTAAAT
GGAGGAAATTAGTAGATTTCAGAGAACTTAATAAGAGAACTCAAGACTTCTG
GGAAGTTCAATTAGGAATACCACATCCCGCAGGGTTAAAAAAGAAAAAATCA
GTAACAGTACTGGATGTGGGTGATGCATATTTTTCAGTTCCCTTAGATGAAG
ACTTCAGGAAGTATACTGCATTTACCATACCTAGTATAAACAATGAGACACC
AGGGATTAGATATCAGTACAATGTGCTTCCACAGGGATGGAAAGGATCACC
AGCAATATTCCAAAGTAGCATGACAAAAATCTTAGAGCCTTTTAAAAAACAAA
ATCCAGACATAGTTATCTATCAATACATGAACGATTTGTATGTAGGATCTGAC
TTAGAAATAGGGCAGCATAGAACAAAAATAGAGGAGCTGAGACAACATCTG
TTGAGGTGGGGACTTACCACACCAGACAAAAAACATCAGAAAGAACCTCCA
TTCCTTTGGATGGGTTATGAACTCCATCCTGATAAATGGACAGTACAGCCTA
TAGTGCTGCCAGAAAAAGACAGCTGGACTGTCAATGACATACAGAAGTTAG
TGGGGAAATTGAATACCGCAAGTCAGATTTACCCAGGGATTAAAGTAAGGC
AATTATGTAAACTCCTTAGAGGAACCAAAGCACTAACAGAAGTAATACCACT
AACAGAAGAAGCAGAGCTAGAACTGGCAGAAAACAGAGAGATTCTAAAAGA
ACCAGTACATGGAGTGTATTATGACCCATCAAAAGACTTAATAGCAGAAATA
CAGAAGCAGGGGCAAGGCCAATGGACATATCAAATTTATCAAGAGCCATTT
AAAAATCTGAAAACAGGAAAATATGCAAGAATGAGGGGTGCCCACACTAAT
GATGTAAAACAATTAACAGAGGCAGTGCAAAAAATAACCACAGAAAGCATAG
TAATATGGGGAAAGACTCCTAAATTTAAACTACCCATACAAAAGGAAACATG
GGAAACATGGTGGACAGAGTATTGGCAAGCCACCTGGATTCCTGAGTGGGA
GTTTGTTAATACCCCTCCTTTAGTGAAATTATGGTACCAGTTAGAGAAAGAA
CCCATAGTAGGAGCAGAAACCTTCTATGTAGATGGGGCAGCTAACAGGGAG
ACTAAATTAGGAAAAGCAGGATATGTTACTAACAAAGGAAGACAAAAGGTTG
TCCCCCTAACTAACACAACAAATCAGAAAACTCAGTTACAAGCAATTTATCTA
GCTTTGCAGGATTCAGGATTAGAAGTAAACATAGTAACAGACTCACAATATG
CATTAGGAATCATTCAAGCACAACCAGATAAAAGTGAATCAGAGTTAGTCAA
TCAAATAATAGAGCAGTTAATAAAAAAGGAAAAGGTCTATCTGGCATGGGTA
CCAGCACACAAAGGAATTGGAGGAAATGAACAAGTAGATAAATTAGTCAGT
GCTGGAATCAGGAAAATACTATTTTTAGATGGAATAGATAAGGCCCAAGATG
AACATTAGTTTTTATGTCGACCTGCAGGGAAAGTTTTATAGGTAGTTGATAG
AACAAAATACATAATTTTGTAAAAATAAATCACTTTTTATACTAATATGACACG
ATTACCAATACTTTTGTTACTAATATCATTAGTATACGCTACACCTTTTCCTCA

FIG. 46

(Figure B_4)

GACATCTAAAAAAATAGGTGATGATGCAACTTTATCATGTAATCGAAATAATA
CAAATGACTACGTTGTTATGAGTGCTTGGTATAAGGAGCCCAATTCCATTAT
TCTTTTAGCTGCTAAAAGCGACGTCTTGTATTTTGATAATTATACCAAGGATA
AAATATCTTACGACTCTCCATACGATGATCTAGTTACAACTATCACAATTAAA
TCATTGACTGCTAGAGATGCCGGTACTTATGTATGTGCATTCTTTATGACATC
GCCTACAAATGACACTGATAAAGTAGATTATGAAGAATACTCCACAGAGTTG
ATTGTAAATACAGATAGTGAATCGACTATAGACATAATACTATCTGGATCTAC
ACATTCACCAGAAACTAGTTAAGCTTGTCTCCCTATAGTGAGTCGTATTAGA
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCT
CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGG
TGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCT
TTCGAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGC
GCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT
CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGA
ACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCG
TTGCTGGCGTTTTTCGATAGGCTCCGCCCCCCTGACGAGCATCACAAAAAT
CGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCG
CTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCT
CATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAG
CTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATC
CGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACT
GGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTG
CTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAG
TATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGG
TAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTT
TGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTG
ATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGG
ATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTA
AAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA
GTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCG
TTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAG
GGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCA
CCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCG
CAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGC
CGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTT
GGCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCA
TTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGT
GCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTAC
TGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAG
TCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCA
ATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTG
GAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT
CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTAC

FIG. 47

(Figure B_5)

TTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAA
AAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTT
CAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATT
TGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGA
AAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAA
AAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGG
TGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTA
AGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTT
GGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACT
GAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAA
ATACCGCATCAGGCGCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGG
GCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGAT
GTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGAC
GTTGTAAAACGACGGCCAGTGAATTGGATTTAGGTGACACTATA

New Psyn II Promoter which controls ADA envelope expression:

TAAAAAATGAAAAAATATTCTAATTTATAGGACGGTTTTGATTTTCTTTTTTTC
TATGCTATAAATAATAAATA

ADA envelope truncated:

ATGAAAGTGAAGGGGATCAGGAAGAATTATCAGCACTTGTGGAAATGGGGC
ATCATGCTCCTTGGGATGTTGATGATCTGTAGTGCTGTAGAAAATTTGTGGG
TCACAGTTTATTATGGGGTACCTGTGTGGAAAGAAGCAACCACCACTCTATT
TTGTGCATCAGATGCTAAAGCATATGATACAGAGGTACATAATGTTTGGGCC
ACACATGCCTGTGTACCCACAGACCCCAACCCACAAGAAGTAGTATTGGAA
AATGTGACAGAAAATTTTAACATGTGGAAAAATAACATGGTAGAACAGATGC
ATGAGGATATAATCAGTTTATGGGATCAAAGCCTAAAGCCATGTGTAAAATT
AACCCCACTCTGTGTTACTTTAAATTGCACTGATTTGAGGAATGTTACTAATA
TCAATAATAGTAGTGAGGGAATGAGAGGAGAAATAAAAAACTGCTCTTTCAA
TATCACCACAAGCATAAGAGATAAGGTGAAGAAAGACTATGCACTTTTCTAT
AGACTTGATGTAGTACCAATAGATAATGATAATACTAGCTATAGGTTGATAAA
TTGTAATACCTCAACCATTACACAGGCCTGTCCAAAGGTATCCTTTGAGCCA
ATTCCCATACATTATTGTACCCCGGCTGGTTTTGCGATTCTAAAGTGTAAAG
ACAAGAAGTTCAATGGAACAGGGCCATGTAAAAATGTCAGCACAGTACAAT
GTACACATGGAATTAGGCCAGTAGTGTCAACTCAACTGCTGTTAAATGGCAG
TCTAGCAGAAGAAGAGGTAGTAATTAGATCTAGTAATTTCACAGACAATGCA
AAAAACATAATAGTACAGTTGAAAGAATCTGTAGAAATTAATTGTACAAGACC
CAACAACAATACAAGGAAAAGTATACATATAGGACCAGGAAGAGCATTTTAT
ACAACAGGAGAAATAATAGGAGATATAAGACAAGCACATTGCAACATTAGTA
GAACAAAATGGAATAACACTTTAAATCAAATAGCTACAAAATTAAAAGAACAA
TTTGGGAATAATAAAACAATAGTCTTTAATCAATCCTCAGGAGGGGACCCAG
AAATTGTAATGCACAGTTTTAATTGTGGAGGGGAATTCTTCTACTGTAATTCA
ACACAACTGTTTAATAGTACTTGGAATTTTAATGGTACTTGGAATTTAACACA

FIG. 48

(Figure B_6)

ATCGAATGGTACTGAAGGAAATGACACTATCACACTCCCATGTAGAATAAAA
CAAATTATAAATATGTGGCAGGAAGTAGGAAAAGCAATGTATGCCCCTCCCA
TCAGAGGACAAATTAGATGCTCATCAAATATTACAGGGCTAATATTAACAAG
AGATGGTGGAACTAACAGTAGTGGGTCCGAGATCTTCAGACCTGGGGGAG
GAGATATGAGGGACAATTGGAGAAGTGAATTATATAAATATAAAGTAGTAAA
AATTGAACCATTAGGAGTAGCACCCACCAAGGCAAAAAGAAGACTGGTGCA
GAGAGAAAAAAGAGCAGTGGGAACGATAGGAGCTATGTTCCTTGGGTTCTT
GGGAGCAGCAGGAAGCACTATGGGCGCAGCGTCAATAACGCTGACGGTAC
AGGCCAGACTATTATTGTCTGGTATAGTGCAACAGCAGAACAATTTGCTGAG
GGCTATTGAGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAA
GCAGCTCCAGGCAAGAGTCCTGGCTGTGGAAAGATACCTAAGGGATCAACA
GCTCCTAGGGATTTGGGGTTGCTCTGGAAAACTCATCTGCACCACTGCTGT
GCCTTGGAATGCTAGTTGGAGTAATAAAACTCTGGATATGATTTGGGATAAC
ATGACCTGGATGGAGTGGGAAAGAGAAATCGAAAATTACACAGGCTTAATAT
ACACCTTAATTGAGGAATCGCAGAACCAACAAGAAAAGAATGAACAAGACTT
ATTAGCATTAGATAAGTGGGCAAGTTTGTGGAATTGGTTTGACATATCAAATT
GGCTGTGGTATGTAAAAATCTTCATAATGATAGTAGGAGGCTTGATAGGTTT
AAGAATAGTTTTTACTGTACTTTCTATAGTAAATAGAGTTAGGCAGGGATACT
CACCATTGTCATTTCAGACCCACCTCCCAGCCCCGAGGGGACCCGACAGG
CCCGAAGGAATCGAAGAAGAAGGTGGAGACAGAGAC

PmH5 promoter (which controls HXB2 gag pol expression):

AAAAATTGAAAATAAATACAAAGGTTCTTGAGGGTTGTGTTAAATTGAAAGC
GAGAAATAATCATAAATA

HXB2 gag pol (with safety mutations, Δ integrase):

ATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGATGGGA
AAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATA
GTATGGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTA
GAAACATCAGAAGGCTGTAGACAAATACTGGGACAGCTACAACCATCCCTT
CAGACAGGATCAGAAGAACTTAGATCATTATATAATACAGTAGCAACCCTCT
ATTGTGTGCATCAAAGGATAGAGATAAAAGACACCAAGGAAGCTTTAGACAA
GATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCACAGCAAGCAGCAGC
TGACACAGGACACAGCAATCAGGTCAGCCAAAATTACCCTATAGTGCAGAA
CATCCAGGGGCAAATGGTACATCAGGCCATATCACCTAGAACTTTAAATGCA
TGGGTAAAAGTAGTAGAAGAGAAGGCTTTCAGCCCAGAAGTGATACCCATG
TTTTCAGCATTATCAGAAGGAGCCACCCCACAAGATTTAAACACCATGCTAA
ACACAGTGGGGGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCA
ATGAGGAAGCTGCAGAATGGGATAGAGTGCATCCAGTGCATGCAGGGCCT
ATTGCACCAGGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAAC
TACTAGTACCCTTCAGGAACAAATAGGATGGATGACAAATAATCCACCTATC
CCAGTAGGAGAAATTTATAAAAGATGGATAATCCTGGGATTAAATAAAATAG
TAAGAATGTATAGCCCTACCAGCATTCTGGACATAAGACAAGGACCAAAAGA
ACCCTTTAGAGACTATGTAGACCGGTTCTATAAAACTCTAAGAGCCGAGCAA

FIG. 49

(Figure B_7)

GCTTCACAGGAGGTAAAAAATTGGATGACAGAAACCTTGTTGGTCCAAAATG
CGAACCCAGATTGTAAGACTATTTTAAAAGCATTGGGACCAGCGGCTACACT
AGAAGAAATGATGACAGCATGTCAGGGAGTAGGAGGACCCGGCCATAAGG
CAAGAGTTTTGGCTGAAGCAATGAGCCAAGTAACAAATTCAGCTACCATAAT
GATGCAGAGAGGCAATTTTAGGAACCAAAGAAAGATTGTTAAGTGTTTCAAT
TGTGGCAAAGAAGGGCACACAGCCAGAAATTGCAGGGCCCCTAGGAAAAA
GGGCTGTTGGAAATGTGGAAAGGAAGGACACCAAATGAAAGATTGTACTGA
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FIG. 50

(Figure B_8)

AATCAGAGTTAGTCAATCAAATAATAGAGCAGTTAATAAAAAAGGAAAAGGT
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FIG. 51

(Figure B_9)

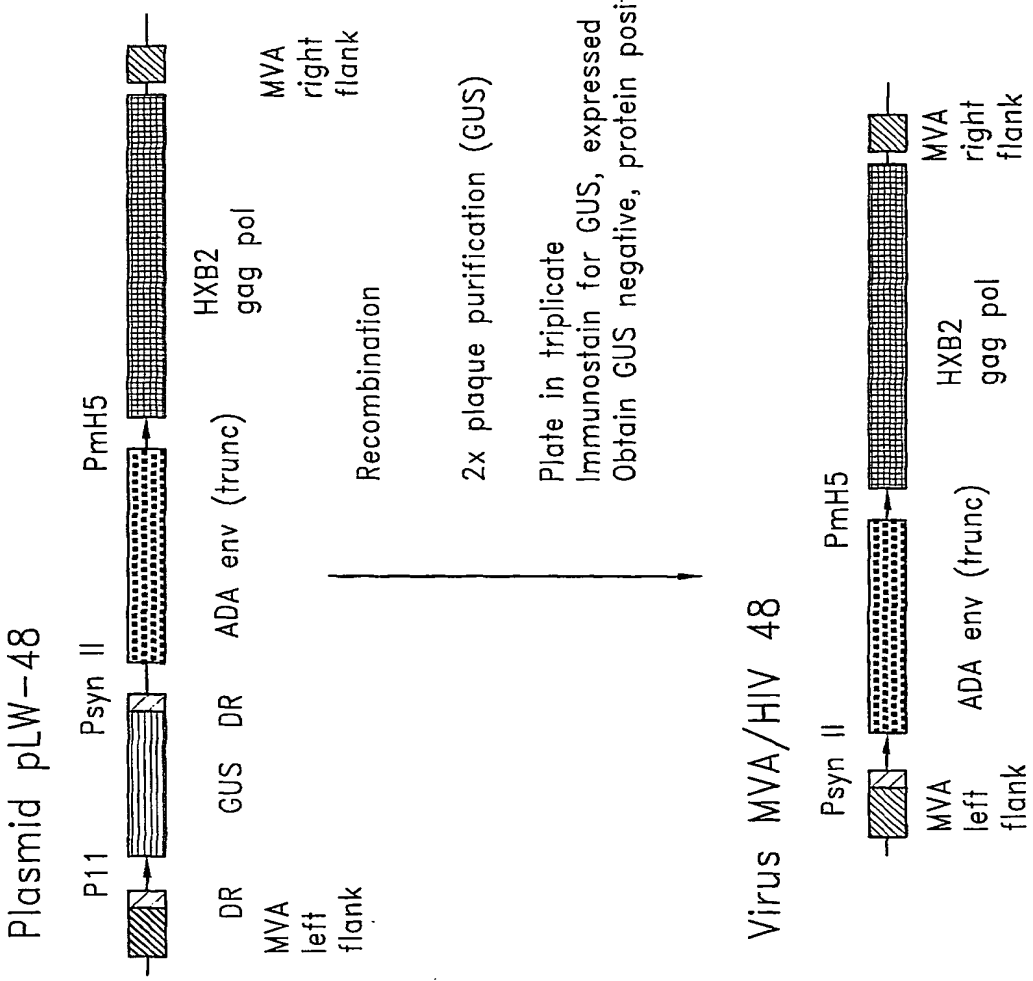

FIG. 52

(Figure C)

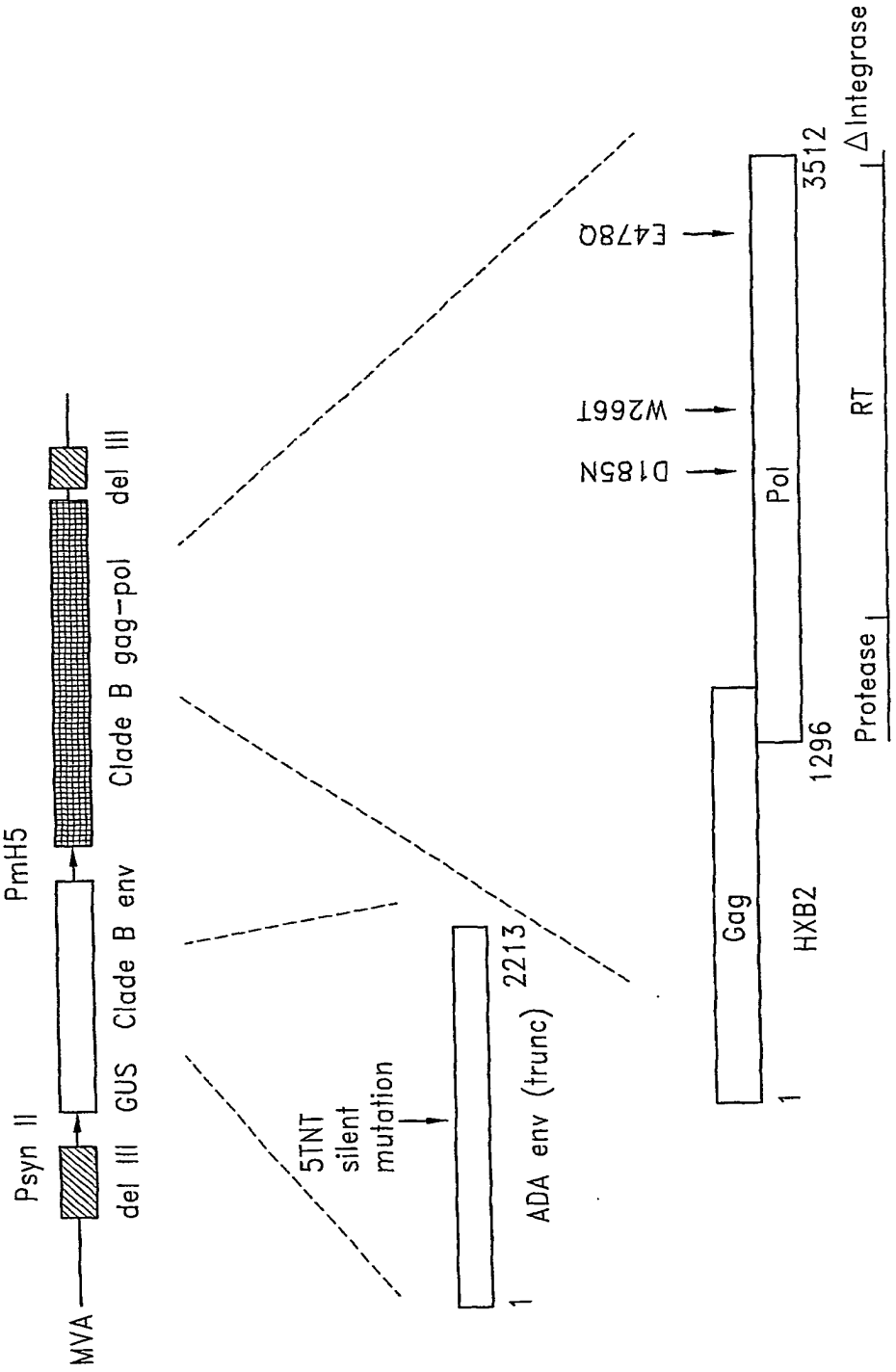

FIG. 53
(Figure D)

Sequence of new Psyn II promoter:

Early part of promoter

Critical region Early start site

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Late part of promoter

TTTGATTTTCTTTTTTTCTATGCTATAAATAATAAATA

FIG. 54

(Figure E)

SEQUENCE LISTING

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Moss, Bernard
Wyatt, Linda
Earl, Patricia

<120> MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

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<150> US 60/274,434
<151> 2001-03-08

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(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

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Declaration under Rule 4.17:
— *of inventorship (Rule 4.17(iv)) for US only*

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

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Previous Correction:
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/072754 A3

(54) Title: MVA EXPRESSING MODIFIED HIV ENVELOPE, GAG, AND POL GENES

(57) Abstract: The invention provides modified virus Ankara (MVA), a replication-deficient strain of vaccinia virus, expressing human immunodeficiency virus (HIV) *env*, *gag*, and *pol* genes.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/06713

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/12, 39/21, 39/275, 39/285 .
US CL : 424/199.1, 204.1, 207.1, 208.1, 232.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/199.1, 204.1, 207.1, 208.1, 232.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	WO 01/47955 A2 (MEDICAL RESEARCH COUNCIL) 5 July 2001(05.07.2001), see entire document.	1-13
Y	US 5,185,146 A (ALTENBURGER) 9 February 1993 (09.02.1999), see entire document.	1-13
Y	OURMANOV et al. Comparative Efficacy of Recombinant Modified Vaccinia Virus Ankara Expressing Simian Immunodeficiency Virus (SIV) Gag-Pol and/or Env in Macaques Challenged with Pathogenic SIV. Journal of Virology, Vol. 74, No. 6, March 2000, pages 2740-2751, see entire document.	1-13
Y	OURMANOV et al. Recombinant Modified Vaccinia Virus Ankara Expressing the Surface gp120 of Simian Immunodeficiency Virus (SIV) Primes for a Rapid Neutralizing Antibody Response to SIV Infection in Macaques. Journal of Virology, Vol. 74, No. 6, March 2000, pages 2960-2965, see entire document.	1-13
Y	GOMEZ et al. Recombinant proteins produced by vaccinia virus vectors can be incorporated within the virion (IMV form) into different compartments. Archives of Virology, Vol. 146, 2001, pages 875-892, see entire document.	1-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
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Date of mailing of the international search report
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Form PCT/ISA/210 (second sheet) (July 1998)

PCT/US02/06713

INTERNATIONAL SEARCH REPORT

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOSS et al. Protein and recombinant MVA immunization and challenge studies with SHIV 89.6. Retroviruses of Human AIDS and Related Animal Diseases, Colloque des Cent Gardes, 12th, Paris, France, Oct. 25-27, 1999 (2000), Meeting Date 1999, pages 105-107, see Abstract.	1-13

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US02/06713

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claim Nos.: 23 and 24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-13

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Claims 23 and 24 are improper multiple dependent claims and therefore are not grouped.

Group I, claim(s) 1-13, drawn to a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41.

Group II, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vif*.

Group III, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpr*.

Group IV, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *tat*.

Group V, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *rev*.

Group VI, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpu*.

Group VII, claim(s) 1 and 14, drawn to pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *nef*.

Group VIII, claim(s) 15, drawn to MVA/HIV-48 comprising SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5.

Group IX, claim(s) 16, drawn to pLW-48 having the sequence of SEQ ID NO:1.

Group X, claim(s) 17, drawn to a plasmid vector having the sequence of SEQ ID NO:1 excluding the HIV *env*, *gag* and *pol* genes.

Group XI, claim(s) 18, drawn to pLW-48 wherein the *env*, *gag* and *pol* genes have a sequence taken from another clade.

Group XII, claim(s) 19, drawn to a poxvirus comprising an m7.5 promoter having the sequence of SEQ ID NO:10.

Group XIII, claim(s) 19, drawn to a poxvirus comprising a Psyn II promoter having the sequence of SEQ ID NO:2.

Group XIV, claim(s) 19, drawn to a poxvirus comprising a Psyn III promoter having the sequence of SEQ ID NO:11.

Group XV, claim(s) 19, drawn to a poxvirus comprising a Psyn IV promoter having the sequence of SEQ ID NO:12.

Group XVI, claim(s) 19, drawn to a poxvirus comprising a Psyn V promoter having the sequence of SEQ ID NO:13.

Group XVII, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41.

Group XVIII, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vif*.

Group XIX, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpr*.

Group XX, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *tat*.

Group XXI, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *rev*.

Group XXII, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpu*.

Group XXIII, claim(s) 20, drawn to a method of boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *nef*.

Group XXIV, claim(s) 21, drawn to a method of inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primed primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41.

Group XXV, claim(s) 21, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vif*.

Group XXVI, claim(s) 21, drawn to a method of inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpr*.

Group XXVII, claim(s) 21, drawn to a method of inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *tat*.

Group XXVIII, claim(s) 21, drawn to a method of inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *rev*.

INTERNATIONAL SEARCH REPORT

Group XXIX, claim(s) 21, drawn to a method of inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpu*.

Group XXX, claim(s) 21, drawn to a method of inducing a boosting CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *nef*.

Group XXXI, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXII, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *vif* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXIII, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *vpr* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXIV, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *tat* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXV, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *vpu* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXVI, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *rev* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXVII, claim(s) 22 and 25, drawn to a method inducing a CD8+ T cell immune response to an HIV Env, Gag or pol antigen in a primate, said method comprising administering to a primate a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol in addition to the HIV gene *nef* and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said primate being previously primed with a nucleic acid encoding said antigen.

Group XXXVIII, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41.

Group XXXIX, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vif*.

Group XL, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpr*.

INTERNATIONAL SEARCH REPORT

Group XLI, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *tat*.

Group XLII claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *rev*.

Group XLIII, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *vpu*.

Group XLIV, claim(s) 26, drawn to a method of making a pharmaceutical composition comprising a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the *env* gene is modified to encode for an HIV Env protein comprised of gp120 and the membrane-spanning ectodomain of gp41. Said virus additionally expresses the HIV gene *nef*.
The inventions listed as Groups I-XLIV do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
Pursuant to 37 C.F.R. 1.475(d), the ISA/US considers that where multiple products and processes are claimed, the main invention shall consist of the first invention of the categoary first mentioned in the claims and the first recited invention of each of the other categories related thereto. Accordingly, the main invention (Group I) comprises the first recited product, a recombinant MVA virus expressing an HIV Env, Gag and Pol and wherein the env gene is modified to encode for an HIV Env protein comprosed of gp120 and the membrane-spanning ectodomain of gp41. Further pursuant to 37 C.F.R. 1.475(d), the ISA/US considers that any feature which the subsequently recited products and methods share with the main invention does not constitute a special technical feature within the meaning of PCT rule 13.2 and that each of such products and methods accordingly defines a separate invention.

Continuation of B. FIELDS SEARCHED Item 3:
STN, EAST, MEDLINE,

search terms: MVA, modified vaccinia Ankara, Env, Gag, Pol, HIV, gp120, gp41, cytoplasmic domain, deletion III, H5-like early/late promoter, vaccinia